Mutational analysis of *Drosophila* STAT92E

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Abbreviations

ATP	adenosine-5´-triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate, toluidine salt
bp	base pair
BSA	bovine serum albumin, fraction V
C-	carboxy
cAMP	cyclic adenosine monophosphate
cDNA	complentary DNA
DIG	digoxygenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleosid triphosphate
DTT	dithiothreitol
EDTA	ethylendiamintetraacetate
EST	expressed sequence tag
Fig.	Figure
(E)GFP	(enhanced) green fluorescent protein
h	hour(s)
HEPES	N-(2-hydroxyethyl)piperazine-N´-2-ethansulfonate
kb	kilobase
kDa	kilo Dalton
min	minute(s)
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium chloride
N-	amino
o/n	over night
ORF	open reading frame
PBS	phosphate buffered saline
rpm	revolutions per minute
RT	room temperature
SDS	sodium-dodecyl-sulfate
sec	second(s)
Tris	tris-hydroxymethyl-aminomethane
UTP	uridine-5´-triphosphate
w	*white*
y	*yellow*

1 Introduction

1.1 Signal Transduction

During the development of complex organisms cells have to undergo both proliferation and differentiation events to finally generate a multi-cellular body composed of various tissues. Each cell of such an organism descends from a single progenitor cell, the zygote. The ability of differentiation, despite the common genome, is based on the temporally and spatially regulated expression of distinct subsets of genes. Cellular identity and perception of the environment is achieved by communication between cells mediated by the sending and receiving of signals. These signals are processed by molecular relay mechanisms that are called signal transduction pathways. One of the known modes of signal transduction includes an extra-cellular signal, the so-called ligand, which binds to its specific transmembrane receptor of a receiving cell. The receptor subsequently gets activated, for example by conformational changes in its cytoplasmic tail. Downstream components are activated in response to these actions. In turn, these components modify cytoplasmic transducers, which carry on the signal and finally activate transcription factors that subsequently alter target gene expression (reviewed in Gerhart, 1999). Despite the multiplicity of cellular processes an organism has to accomplish, only 17 different signal transduction pathways are known so far (reviewed in Gerhart, 1999). The wide variety of actions required for proper development of an organism can therefore only be achieved by crosstalk of different pathways, modulation of the intensity of the received signal and the history of a cell, the so-called cell´s competence (Pires-da Silva and Sommer, 2003).

1.2 Protein modifications

Signal transduction is often mediated by altering the state of signal transducing proteins by introducing post-translational modifications of amino acid side chains. Many of these proteins, including tyrosine kinases, histones and RNA polymerase II, are not only modified at a single amino acid, but display modifications at multiple sites (reviewed in Yang, 2005). Over 200 different forms of such modifications are known so far (reviewed in Yang, 2005).

1.2.1 Protein phosphorylation

The major protein modification associated with signal transduction is phosphorylation. Protein kinases transduce the incoming signal by transferring phosphate groups to the hydroxyl side chain of tyrosine (Eckhart *et al.*, 1979), serine or threonine residues (reviewed in McCubrey *et al.*, 2000) of target proteins. Tyrosine phosphorylation is often required for signal transduction as shown for the Receptor Tyrosine Kinase (RTK) pathway and cytokine signaling (see also 1.4). Additionally, serine phosphorylation, for example, is required for full transcriptional activity of the Signal Transducer and Activator of Transcription (STAT) 1 protein (Eilers *et al.*, 1995). Furthermore, serine and threonine phosphorylation of histones contributes to transcriptional activation by chromatin remodeling (reviewed in Spencer and Davie, 1999).

Phosphorylation is a reversible process. Phosphatases counteract the action of kinases by removing the introduced phosphate group of the modified proteins. As such, the change in protein function caused by phosphorylation can be regulated quickly and reversibly. The significance of the function of kinases and phosphatases is reflected in their large number in various genomes (Arena *et al.*, 2005). For the human genome, at least 518 different genes coding for kinases are proposed constituting about 1.7% of all predicted genes (Manning *et al.*, 2002). Additionally, about 180 phosphatases are known so far. The importance of both kinases and phosphatases becomes also apparent by mutations in these enzymes, which have frequently been associated with the onset of cancer (Arena *et al.*, 2005).

1.2.2 Protein methylation

Another reversible post-translational modification involved in signal transduction is protein methylation of arginine, lysine, histidine, proline side chains and the C-terminal carboxyl group of the amino acid backbone. In general, the enzymes catalyzing methylation reactions are called methyltransferases. S-adenosylmethionine (SAM) provides the methyl group in the enzymatic reaction (reviewed in Chiang *et al.*, 1996). Transfer of its methyl group results in S-adenosylhomocysteine and the methylated target.

Reversible carboxy-methylation of glutamate side chains in receptor-transducer proteins, for example, is implicated in bacterial chemotaxis. Mutants lacking the methylating and demethylating enzymes exhibit an aberration in their motility pattern (reviewed in Chiang *et al.*, 1996). Additionally, methylation of several lysine and arginine side chains in histones was

shown to contribute to both positive and negative regulation of transcription (reviewed in Stallcup, 2001; Lee *et al.*, 2005).

Arginine methylation

Recently, arginine methylation and its role in signal transduction and transcriptional control has come into focus (reviewed in Stallcup, 2001). Protein Arginine Methyltransferases (PRMT) catalyze this modification by transferring methyl groups from the donor molecule SAM to the guanidino group of arginine residues of protein substrates. Arginine is a positively charged amino acid and often plays an important role in protein-protein interactions frequently mediated by hydrogen bonding.

Methylation does not alter the charge of the arginine side chain, but rather increases its bulkiness and hydrophobicity. Arginine methylation mediated by PRMTs can have both negatively and positively regulating effects on protein function and protein-protein interactions (reviewed in Bedford and Richard, 2005; Boisvert *et al.*, 2005). Recently, over 200 new proteins were identified that are putatively arginine methylated implicating this post-translational modification in many cellular processes including signal transduction, DNA repair, transcription, translation and apoptosis (Boisvert *et al.*, 2003). The Co-activator Associated Arginine Methyltransferase (CARM) 1 methylates arginine residues in the CREB Binding Protein (CBP) and p300, which are transcriptional co-activators for a large number of transcription factors including cAMP Responsive Element Binding Protein (CREB), STAT1 and nuclear receptors (Chevillard-Briet *et al.*, 2002). Methylation of CBP/p300 occurs in distinct domains. Depending on the methylation site, this modification has an inhibitory effect on CREB mediated somatostatin expression by destabilizing the domain mediating CREB recruitment (Xu *et al.*, 2001), whereas methylation of a distinct arginine residue was shown to have a positive influence on reporter gene transcription mediated by nuclear receptors (Chevillard-Briet *et al.*, 2002). Arginine methylation of the transcription factor STAT1 has been reported to modulate Interferon (IFN) α/β signaling by affecting the interaction between STAT1 and the negative regulator Protein Inhibitor of Activated STAT (PIAS) (Mowen *et al.*, 2001).

1.3 Protein domains recognizing modified amino acid residues

Transducing cellular signaling events by changing the activity of proteins through post-translational modifications requires the ability to distinguish between unmodified and modified residues. Various protein modules exist which are known to bind modified amino acid side chains with high affinity (reviewed in Yang, 2005). Among these modules the Src-homology 2 (SH2) domain (Sadowski *et al.*, 1986) recognizes phosphorylated tyrosine residues of other proteins. Essential for binding of these phosphorylated tyrosine side chains is a highly conserved arginine residue, located at the base of the binding pocket. This arginine is conserved in the SH2 domain of various proteins, such as the kinases c-Src and c-Abl and the phospholipase PLCγ (reviewed in Koch *et al.*, 1991). This residue is also evolutionary conserved within protein families in species ranging from *Dictyostelium* to human as shown for the transcription factor STAT (Kawata *et al.*, 1997). At the edge of the binding pocket additional specific contacts are made between the SH2 domain and the tyrosine phosphorylated protein. These contacts are formed between various amino acid side chains of the SH2 domain and three flanking residues C-terminal to the phosphorylated tyrosine. The third residue is bound in the so-called phospho-tyrosine+3 pocket. In particular, this interaction contributes to the binding specificity of different SH2 domain containing proteins to tyrosine phosphorylated protein binding partners (Branden and Tooze, 1999).

The interactions between phosphorylated and SH2 domain containing proteins are crucial events in signal transduction cascades, which use tyrosine phosphorylation to modify the function of pathway components (reviewed in Pawson, 2004).

1.4 The JAK/STAT signal transduction pathway

A signal transduction pathway using the principle of tyrosine phosphorylation and subsequent SH2 domain mediated recognition is the Januskinase (JAK) / Signal Transducer and Activator of Transcription (STAT) cascade. Numerous studies have led to the development of a canonical model of signal transduction (Fig. 1.1; reviewed in O'Shea *et al.*, 2002; Rawlings *et al.*, 2004b). In this model binding of an extra-cellular ligand to its transmembrane receptor results in receptor dimerization or conformational changes of pre-dimerized receptors (Heinrich *et al.*, 2003). This way, the receptor associated JAK proteins are juxtaposed to each other, resulting in mutual phosphorylation, activation and subsequent phosphorylation of tyrosine residues of the receptors.

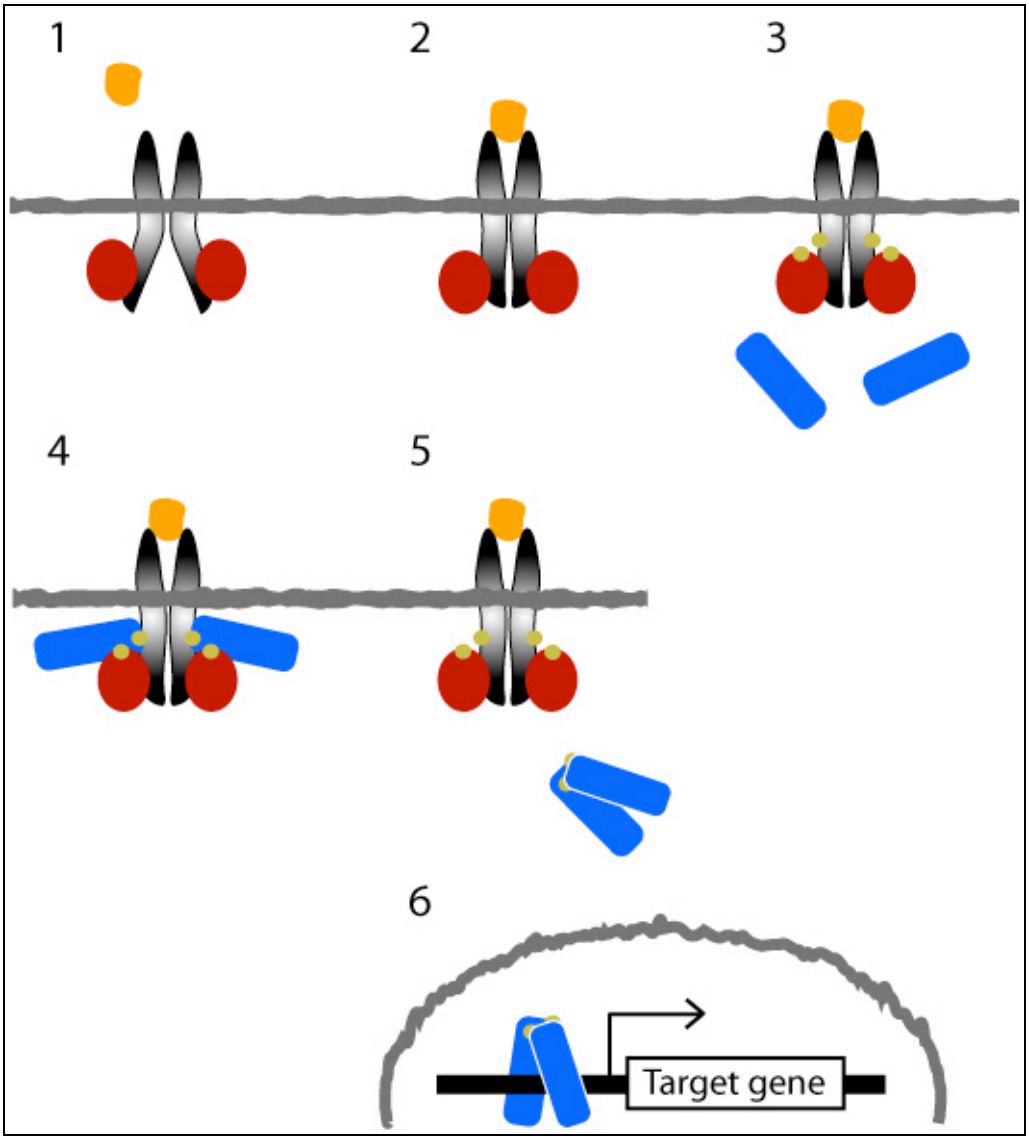

Fig. 1.1: Model of signal transduction by the JAK/STAT pathway (modified after O'Shea *et al.*, 2002). (1) Pre-dimerized receptors (black) and associated JAK proteins (red). **(2)** Ligand (orange) binding to receptors results in conformational changes in the receptors, juxtaposing the JAK proteins to each other. **(3)** JAK proteins transfer a phosphate group (yellow) to tyrosine residues of JAKs and receptors. **(4)** This creates docking sites for STAT proteins (blue). At the receptor-JAK complex STAT proteins are phosphorylated at a conserved tyrosine residue by JAKs. **(5)** After phosphorylation STATs dissociate from the receptor complex and form homo- or heterodimers. **(6)** These dimers translocate to the nucleus, where they bind to palindromic DNA recognition sites present within promoters of pathway target genes and subsequently activate the expression of these genes.

The phosphorylated receptor-JAK complex acts as a docking site for the SH2 domains of cytosolically localized STAT molecules. After recruitment to the receptor-JAK complex, the STAT proteins are themselves phosphorylated at an evolutionary conserved C-terminal tyrosine residue by the JAK proteins. These activated STAT molecules form homo- or hetero-dimers, using their SH2 domains to bind the phosphorylated dimerization partner. The

resulting STAT dimers enter the nucleus by an importin α-5 and Ran dependent mechanism (Rawlings *et al.*, 2004b), where they bind to palindromic DNA recognition sites present within the promoters of pathway target genes. Following DNA binding, STAT factors then stimulate transcription via direct or indirect interaction with the basal transcription machinery (reviewed in Darnell *et al.*, 1994).

1.4.1 JAK/STAT signaling in mammals

In mammals the JAK/STAT pathway is a very complex system with a multiplicity of participating components. More than 40 different ligands including cytokines and growth factors are known (reviewed in Kisseleva *et al.*, 2002). A corresponding variety of different receptors or combinations of these receptors bind these ligands (Hirano *et al.*, 1997; Onishi *et al.*, 1998). All different signals are transduced by only four receptor-associated tyrosine kinases, the JAK proteins JAK1-3 and Tyrosine Kinase (TYK) 2 (reviewed in Yamaoka *et al.*, 2004), and seven different STAT proteins, STAT1-4, STAT5A, STAT5B and STAT6 (reviewed in Brierley and Fish, 2005).

The JAK/STAT signal transduction cascade was originally identified as the primary mediator of IFNα and IFNγ induced signaling (reviewed in Darnell *et al.*, 1994; Rawlings *et al.*, 2004b). Now it is known that a wide array of cytokines and growth factors, together with their respective receptors, use the JAK/STAT cascade for signal transduction. As expected, given the range of signals and receptors being used, JAK/STAT signaling plays an important role in many different processes. Among others, it affects proliferation, differentiation, migration and apoptosis of cells. Additionally, it is crucial for hematopoiesis, the immune response, mammary gland development and lactation, adipogenesis and sexually dimorphic growth (reviewed in Rawlings *et al.*, 2004b). Multiple diseases related to these processes result from mutations in components of the JAK/STAT pathway (Schindler, 2002). Particularly, various blood cell malignancies including leukaemias, lymphomas and multiple myelomas caused by JAK/STAT mis-regulation highlight the central role of JAK/STAT signaling in hematopoiesis (Valentino and Pierre, 2006).

Negative regulation of the JAK/STAT pathway

JAK/STAT signaling is involved in multiple processes, and mis-regulation often has severe effects as outlined above. Therefore, JAK/STAT activity has to be tightly controlled at

multiple levels to ensure appropriate and controlled cellular responses (reviewed in Wormald and Hilton, 2004). Negative regulation of JAK/STAT activity is mediated by several classes of proteins, three of which are particularly well studied (reviewed in Carbia-Nagashima and Arzt, 2004; Wormald and Hilton, 2004). Among them, the PIAS family of proteins can bind to activated STAT dimers, thus blocking STAT DNA-binding activity. Furthermore, PIAS proteins have been shown to promote STAT1 SUMOylation, thereby inhibiting STAT1 mediated transcription (Liu *et al.*, 1998; Rogers *et al.*, 2003; Ungureanu *et al.*, 2003). In addition, protein phosphatases play an important role in negative regulation of JAK/STAT activity. In particular, members of the Protein Tyrosine Phosphatases (PTP) family of proteins reverse the activity of the JAK kinases, with SH2 containing protein tyrosine phosphatase (SHP) 1 as the first identified JAK/STAT phosphatase (David *et al.*, 1995; Haque *et al.*, 1998). Finally, the Suppressors Of Cytokine Signaling (SOCS) family has eight members in mammals, SOCS1-7 and Cytokine-Inducible SH2-Containing Protein (CIS) (reviewed in Kile and Alexander, 2001; Larsen and Ropke, 2002). At least three mechanisms for negative regulation of JAK/STAT activity by SOCS proteins are known so far. These proteins can bind via their SH2 domain to phosphorylated receptors, thus blocking the recruitment of STAT proteins to the receptor complex (Yoshimura *et al.*, 1995). Additionally, JAK kinase activity can be specifically inhibited by SOCS binding to JAK proteins (Yasukawa *et al.*, 1999). Furthermore, an interaction of SOCS proteins with the elongin B/C complex was reported. This complex can bind an E3-like ubiquitin ligase suggesting the ubiquitination of JAKs and receptors to target them to the proteasome for degradation (Zhang *et al.*, 1999). SOCS1 - 3 and CIS expression is dependent on JAK/STAT activity. JAK/STAT signaling thus mediates the expression of signaling inhibitors to down-regulate its own activity. Hence, these SOCS proteins act together with the JAK/STAT pathway components in a classical negative feedback loop (reviewed in Larsen and Ropke, 2002).

1.4.2 JAK/STAT signaling in *Drosophila melanogaster*

The JAK/STAT pathway in *Drosophila melanogaster* is the most intensively studied example of an invertebrate JAK/STAT signaling cascade (reviewed in Luo and Dearolf, 2001; Hombría and Brown, 2002; Arbouzova and Zeidler, 2006). A single STAT-like gene termed *stat92E* is known (Hou *et al.*, 1996; Yan *et al.*, 1996). Additionally, a single JAK homologue called *hopscotch* (*hop*) was identified (Binari and Perrimon, 1994). The secreted glycoprotein Unpaired (Upd) (Harrison *et al.*, 1998) and two closely related homologues Upd2 (Gilbert *et*

al., 2005; Hombría *et al.*, 2005) and Upd3 (Agaisse *et al.*, 2003) are the only pathway ligands known so far. They do not show sequence similarity to any vertebrate cytokine. However, they show structural features that are conserved throughout evolution and are reminiscent of mammalian cytokines (Boulay *et al.*, 2003). The receptor Domeless (Dome) (Brown *et al.*, 2001) is the only JAK/STAT receptor described in *Drosophila*. It shows weak similarities to the vertebrate interleukin-6 receptor. Furthermore, the predicted gene *CG14225* shows similarities to *Dome*, but no functional analysis has yet been done (Hombría and Brown, 2002). Taken together, all components known from the vertebrate pathway are also present in *Drosophila*, with the big advantage of much less redundancy.

In addition to the homology between vertebrate and *Drosophila* JAK/STAT pathway components, a number of developmental roles for JAK/STAT signaling have also been conserved (reviewed in Luo and Dearolf, 2001; Hou *et al.*, 2002; Arbouzova and Zeidler, 2006). These include its requirement for the development and differentiation of *Drosophila* hematopoietic cells (Hanratty and Dearolf, 1993; Harrison *et al.*, 1995; Luo *et al.*, 1995; Luo *et al.*, 1997). Gain-of-function mutants of the kinase Hopscotch were shown to cause melanotic tumor formation and abnormalities in blood cell development (reviewed in Luo and Dearolf, 2001). JAK/STAT activity is also required for the response to bacterial infection (Agaisse *et al.*, 2003; Agaisse and Perrimon, 2004). In addition, it functions in regulating cellular proliferation in wing imaginal discs (Mukherjee *et al.*, 2005). Furthermore, loss of JAK/STAT signaling causes embryonic segmentation defects (Perrimon and Mahowald, 1986; Binari and Perrimon, 1994; Hou *et al.*, 1996; Harrison *et al.*, 1998). JAK/STAT activity is also required for the induction of *trachealess* (*trh*) expression (Brown *et al.*, 2001), which is essential for tracheal morphogenesis (Isaac and Andrew, 1996; Wilk *et al.*, 1996). Furthermore, fore- and hindgut development (Johansen *et al.*, 2003; Josten *et al.*, 2004), the maintenance of stem cell renewal and development of both the adult testis and ovary (Hombría and Brown, 2002; Decotto and Spradling, 2005) have been shown to require JAK/STAT signaling. Development of the *Drosophila* eye is also dependent on JAK/STAT activity (Luo *et al.*, 1999; Zeidler *et al.*, 1999a; Zeidler *et al.*, 1999b), as disruption of signaling results in a small eye phenotype (Luo *et al.*, 1999).

Negative regulation of the JAK/STAT pathway in *Drosophila*

Negative regulators of JAK/STAT activity characterized in vertebrate systems are also present and functional in *Drosophila* as shown for a PIAS homologue (Mohr and Boswell,

1999; Betz *et al.*, 2001; Hari *et al.*, 2001). Additionally, genome-wide RNAi screens have identified Ptp61F among several other PTPs as negative regulator of *Drosophila* JAK/STAT signaling (Baeg *et al.*, 2005; Müller *et al.*, 2005). Furthermore, a family of SOCS proteins was also identified to regulate JAK/STAT activity (Callus and Mathey-Prevot, 2002; Karsten *et al.*, 2002; Rawlings *et al.*, 2004a). Three SOCS proteins are known in *Drosophila*: SOCS16D, SOCS36E and SOCS44A. It was shown that SOCS36E suppresses *Drosophila* JAK/STAT activity (Callus and Mathey-Prevot, 2002; Müller *et al.*, 2005). *socs36E* is a transcriptional target of JAK/STAT signaling. Loss of pathway activity resulted in disruption of *socs36E* expression, whereas ectopic pathway activation induced ectopic *socs36E* expression (Karsten *et al.*, 2002). This indicates that SOCS36E is part of a negative feedback loop as shown for vertebrate SOCS proteins (reviewed in Larsen and Ropke, 2002). SOCS44A is also a negative regulator of *Drosophila* JAK/STAT signal transduction, but its expression is regulated independently of JAK/STAT signaling (Rawlings *et al.*, 2004a).

1.5 Signal Transducer and Activator of Transcription

The family of STAT proteins represents a central component of the JAK/STAT pathway (reviewed in Brierley and Fish, 2005).

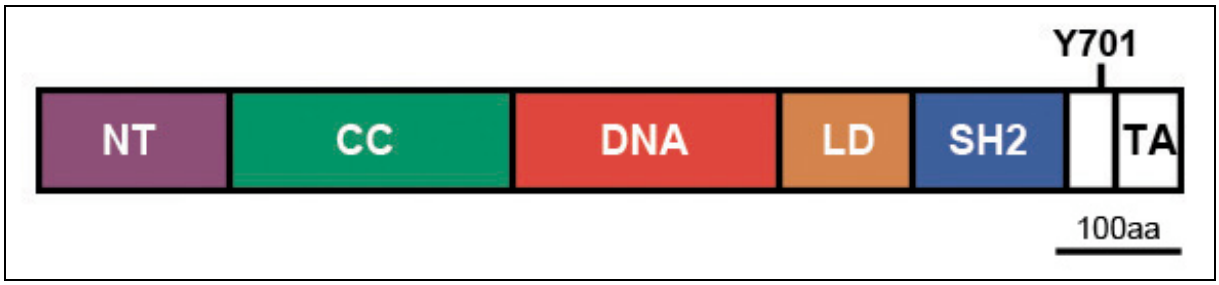

Fig. 1.2: Conserved domains of STAT proteins (modified after Chen *et al.*, 1998). STAT1 is shown as example. Size bar represents 100 amino acids. The N-terminal domain (NT) of STAT proteins is involved in protein-protein interactions. The coiled-coil (CC) and the SH2 domain (SH2) have been implicated in receptor binding (Mikita *et al.*, 1998; Zhang *et al.*, 2000). Furthermore, the SH2 domain is essential for STAT dimerization (Shuai *et al.*, 1994). The DNA binding domain (DNA) as well as the Linker (LD) and the SH2 domain have been implicated in DNA binding (Mikita *et al.*, 1998; Yang *et al.*, 2002). The Transactivation domain (TA) is involved in transcriptional activation (Müller *et al.*, 1993). Y701 of STAT1 represents the conserved tyrosine residue, which is phosphorylated by JAK proteins and essential for STAT activation (Shuai *et al.*, 1993b).

STAT proteins share a common protein structure (Fig. 1.2), which includes an N-terminal protein-protein interaction domain, followed by a coiled-coil domain involved in receptor binding (Zhang *et al.*, 2000). A DNA binding domain (Horvath *et al.*, 1995) is connected to a SH2 domain via a linker domain, which is also implicated in DNA binding (Yang *et al.*, 2002). The SH2 domain is responsible for dimerization and binding to the receptor complex (Shuai *et al.*, 1994). A transcriptional activation domain is located at the C-terminus (Müller *et al.*, 1993). Between these two domains a highly conserved tyrosine residue is situated. This tyrosine is the phosphorylation target of JAK proteins as it was first suggested for STAT1 and JAK1 (Shuai *et al.*, 1993b).

1.5.1 STAT function and control mechanisms of its activation

Signal transduction mediated by STAT proteins plays a central role in developmentally important processes (reviewed in O'Shea *et al.*, 2002). Therefore, it is not surprising that multiple regulatory mechanisms have evolved to control STAT activity. As outlined above, phosphorylation of a highly conserved tyrosine residue within the C-terminus of all STAT proteins is the key factor in their activation. This modification is required for the formation of STAT dimers via interactions between their SH2 domains and opposing phospho-tyrosine residues (Shuai *et al.*, 1994). In addition to tyrosine phosphorylation, other post-translational modifications have been shown to regulate STAT function. These modifications include phosphorylation of a C-terminal serine residue of STAT1 increasing the potential of STAT1 to activate transcription (Eilers *et al.*, 1995). Serine phosphorylation was also shown for STAT3, STAT4, STAT5a and STAT5b (reviewed in Brierley and Fish, 2005). Additionally, PIAS mediated SUMOylation of STAT1 is implicated in the down-regulation of transcriptional activity (Rogers *et al.*, 2003; Ungureanu *et al.*, 2003). STAT1 and STAT3 lysine acetylation was reported to stimulate sequence-specific DNA binding and transactivation activity (O'Shea *et al.*, 2005; Wang *et al.*, 2005; Yuan *et al.*, 2005; Kramer *et al.*, 2006). Furthermore, a number of contradictory reports have been published regarding the potential methylation of a conserved N-terminal arginine residue of STAT1 and STAT6 affecting protein-protein interactions or transcriptional regulation (Mowen *et al.*, 2001; Chen *et al.*, 2004; Meissner *et al.*, 2004; Komyod *et al.*, 2005). However, the presence of these modifications in other STAT molecules, their roles *in vivo* and their contribution to the overall level of STAT activity are less clear and still subject of research.

Analysis of several artificially generated mutant STAT proteins has contributed to the understanding of STAT function (Mikita *et al.*, 1998; Bromberg *et al.*, 1999; Ariyoshi *et al.*, 2000; Daniel *et al.*, 2000; Zhang *et al.*, 2000; Yang *et al.*, 2002; Meyer *et al.*, 2004; Liddle *et al.*, 2006). On the basis of these results, models for STAT protein action concerning receptor binding, phosphorylation, dimerization, DNA binding and transcriptional activation have been developed. The coiled-coil and SH2 domain, for example, have been implicated in receptor binding (Mikita *et al.*, 1998; Zhang *et al.*, 2000). Additionally, mutations in the DNA binding domain, as well as in the linker and SH2 domain, have been shown to affect DNA binding (Mikita *et al.*, 1998; Yang *et al.*, 2002). Furthermore, analysis of constitutive active STAT variants highlighted the essential role of a conserved tyrosine residue, phosphorylated by JAK proteins, for STAT function. Mutation of this residue completely abolished the transcriptional activity in the context of these constitutively active proteins (Bromberg *et al.*, 1999; Ariyoshi *et al.*, 2000; Daniel *et al.*, 2000; Liddle *et al.*, 2006).

Structural analysis described that non-phosphorylated STAT1 also exists in dimers (Chen *et al.*, 2003; Mao *et al.*, 2005; Zhong *et al.*, 2005). Dimerization of these non-active STAT1 proteins is not mediated by their SH2 domain, but depends on interactions between the N-terminal domains and also between the coiled-coil and the DNA binding domains of STAT1 dimer partners. It was proposed that tyrosine phosphorylation induces structural rearrangements resulting in the active dimeric form, which is capable of inducing target gene expression. Another conformational rearrangement presenting the phosporylated tyrosine to nuclear phosphatases for dephosphorylation is proposed to be a prerequisite for STAT inactivation (Zhong *et al.*, 2005).

1.5.2 *Drosophila* STAT92E

In *Drosophila* only a single STAT gene is known, *stat92E* (Hou *et al.*, 1996; Yan *et al.*, 1996). Four different mRNAs are transcribed, which are translated into three different proteins (Fig. 1.3). Two transcripts, differing in their transcription start sites and 5′-UTRs, give rise to the same protein of approximately 87 kDa. Additionally, one closely related protein with an insertion of seven additional residues is translated from an alternatively spliced mRNA. Furthermore, a N-terminal truncated protein (ΔNSTAT92E) of approximately 72 kDa is translated lacking 134 residues of the N-terminal domain. This variant acts as a negative regulator of JAK/STAT signaling. It was shown that over-expression of

ΔNSTAT92E is sufficient to suppress JAK/STAT-dependent transcriptional activation of the pair-rule gene *even-skipped in vivo* (Henriksen *et al.*, 2002).

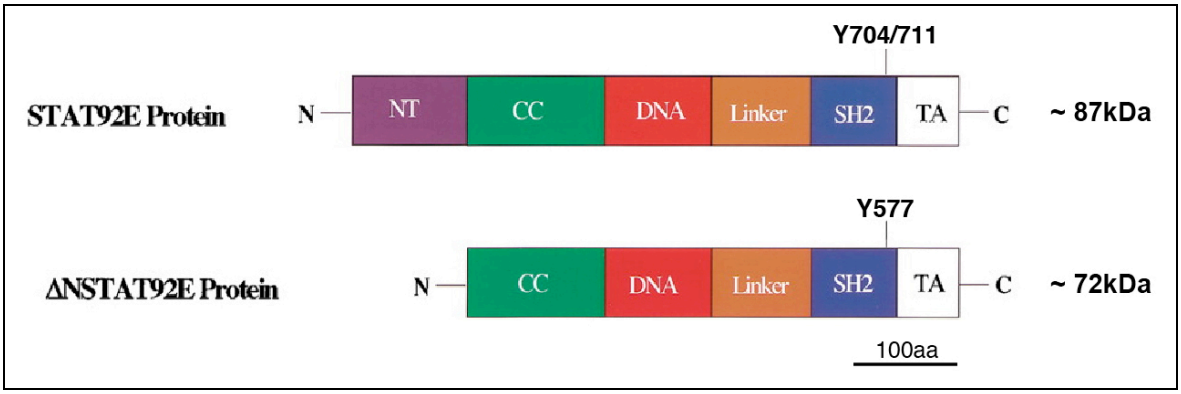

Fig. 1.3: Protein structure of *Drosophila* STAT92E splice variants (modified after Henriksen *et al.*, 2002). Two transcripts differing in the 5´-UTR give rise to identical proteins of ~87kD. Additionally, a protein, in which seven additional residues are inserted after residue 698 via a differential splice event between two exons, is translated. Furthermore, a N-terminal truncated version ΔNSTAT92E of ~72 kDa is also translated. This protein lacks 134 amino acids of the N-terminal domain (NT). Y704/711 and Y577 represent the conserved tyrosine residue, essential for STAT activation (Shuai *et al.*, 1993b). Size bar represents 100 amino acids. CC: Coiled-coil domain, DNA: DNA binding domain, LD: Linker domain, SH2: SH2 domain, TAD: Transactivation domain.

In vertebrates seven different STAT proteins participate in JAK/STAT signaling. Control of overall JAK/STAT activity involves post-translational modifications and interactions of different STAT proteins with co-regulators (reviewed in Brierley and Fish, 2005). However, STAT92E is sufficient to regulate multiple processes in *Drosophila*, which are regulated by different STAT proteins in vertebrates. It was shown that STAT92E exerts opposing roles in cellular proliferation analogous to the pro- and anti-proliferative roles of vertebrate STAT3 and STAT1 (Mukherjee *et al.*, 2005). Therefore, it is very likely that the regulation and control of *Drosophila* STAT92E activity requires a complex system of interactions with multiple co-regulators. One example is the repression of JAK/STAT-dependent expression of the genes *trh*, *ventral veins lacking* and *knirps*, which is mediated by DNA binding of the repressor Ken & Barbie to a subset of STAT92E recognition sites (Arbouzova *et al.*, 2006). Furthermore, regulation of STAT92E activity by post-translational modifications, in addition to tyrosine phosphorylation, most likely also plays an important role for the control of *Drosophila* JAK/STAT signaling.

For the better understanding of the regulatory mechanisms controlling STAT activity *in vivo*, this study characterized *Drosophila* STAT92E protein function by mutational analysis. Loss-

of- and gain-of-function alleles of STAT92E were generated and analyzed for their function in cell culture and *in vivo* assays. This way, residues have been identified that are involved in the regulation of STAT92E sub-cellular localization and DNA binding activity and are also important for STAT92E function as a transcriptional activator.

2 Material & Methods

2.1 Solutions

Table 2-1: Composition of solutions used in this study.

AP buffer	100 mM NaCl, 50 mM $MgCl_2$, 0.1M Tris pH 9.5, 0.1% Tween20
Binding buffer	20 mM HEPES pH 7.9, 1.4 mM $MgCl_2$, 40 mM KCl, 0.1 mM EGTA, 5% Glycerol, 0.5 mM DTT, 1 mM PMSF, 50 mg/ml Poly-dI-dC
Cell resuspension buffer	40 mM HEPES-KOH pH 7.9, 0.4 M KCl, 10% glycerol, 1 mM DTT, 0.1 mM PMSF
Cell rinse buffer	40 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.15M NaCl
EB buffer	10 mM Tris-Cl pH 8.5
Extraction buffer	0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl pH 9.0, 50 mM EDTA, 0.5% SDS
Hybe	50% formamide, 5x SSC, 0.2 mg/ml sonicated salmon testis DNA, 0.1 mg/ml tRNA, 50 µg/ml heparin
Hybe B	50% formamide, 5x SSC
Lysis buffer (for EMSA)	10 mM HEPES pH 7.9, 10 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100, 50% Glycerol, 1 mM DTT, 1 mM Na-O-vanadate, 1 mM PMSF
PBS	130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4
PBT	PBS with 0.1% Tween20 or 0.1% Triton X-100
RIPA buffer	50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 2.5 mM Na-O-vanadate, 0.1% NP-40, 10% glycerol, protease inhibitors (Complete EDTA-free, Roche Diagnostics, Mannheim)
RNA fixative	10% paraformaldehyde in PBS, 50 mM EGTA (pH 7.0)
4x SDS-gel buffer	1.5 M Tris-base, 0.4% SDS (pH 8.8)
10x SDS-running buffer	0.4 M Tris-base, 1.9 M glycin, 0.5% SDS
0.5x TBE buffer	44.5 mM Tris-base, 44.5 mM boric acid, 1 mM EDTA (pH 8.0)
TE buffer	10 mM Tris-HCl pH 8.0, 1 mM EDTA
Transfer buffer	0.25 M Tris-base, 0.2 M glycin, 20% methanol

2.2 Bacterial culture

Bacterial cells of *E. coli* strain XL1-blue (Stratagene, Amsterdam, The Netherlands) were grown either in LB^+ medium with vigorous shaking (300 rpm) or on agar plates at 37°C.

Solutions:

LB^- (per 1l of medium)	10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl (pH 7.0), autoclaved 45 min at 120°C
LB^+	LB^- with 50 µg/ml ampicillin or 34 µg/ml chloramphenicol
LB-agar	15g agar-agar, 1l of LB^-, autoclaved and stored at RT

To prepare plates LB-agar was melted, cooled down approximately to 50°C and supplemented with antibiotics to the same final concentration as in LB^+.

2.3 Nucleic acid manipulation

2.3.1 Molecular cloning

In the genomic region of *socs36E* (GenBank Scaffold: AE003657 & AE003658) fragments containing putative enhancer elements with potential STAT92E binding sites were amplified by PCR (see 2.3.2). To do so, sequence-specific primers for these regions were used, also introducing restriction sites for subsequent cloning (Table 2-2). After restriction (see 2.3.4) these fragments were sub-cloned (see 2.3.5) into the similar cut pW8ßE (see Table 2-4) or the pGL3-Promoter vector (Promega, Mannheim) to generate *socs36E-lacZ* or *socs36E(I)-luc* constructs as reporter for JAK/STAT activity, respectively.

Fusion proteins, termed STAT92E-GFP, were generated for expression in *Drosophila* cells and as a tool to visualize JAK/STAT activity on the basis of STAT92E sub-cellular localization. The fusion protein consists of the full length *stat92E* ORF (Splice form CG4257-RC; FlyBase, 2003) amplified with sequence-specific primers from STAT92E cDNA (Hou *et al.*, 1996) and trimmed with restriction endonucleases *Asp*718 and *Bam*HI. EGFP-fused constructs were produced in two steps: the *stat92E* ORF was first cloned into the pBS-EGFPB vector (M. Zeidler, unpublished) and then re-cloned into the pUAST vector (Brand and Perrimon, 1993). pBS-EGFPB is a cassette based cloning system designed to generate C-terminal fusions with EGFP (Clontech, Saint-Germain-en-Laye, France) and was generated by amplifying EGFP with sequence-specific primers. The PCR products were then trimmed

with *Bam*HI and *Xba*I before cloning into a similarly cut pBS (KS+) vector (Stratagene, Amsterdam, The Netherlands). The resulting EGFP coding region includes a stop codon. The in frame cloning of STAT92E into pBS-EGFPB generates the amino acids N and P not originally present in either protein. pBS-STAT92E-GFP and pUAST-STAT92E-GFP were generated by M. Zeidler and have been described recently (Karsten *et al.*, 2006). These constructs were used as a basis to generate several STAT92E-GFP mutants. Point mutations in STAT92E-GFP were introduced by site-directed mutagenesis (see 2.3.3).

Table 2-2: Schemes for molecular cloning.

Construct	Primers	Template	Restriction sites in polylinker
socs36E(I)-lacZ	GTTAGGTACCGGGTCGCAGTATCGTTGGCG CGAAGGATCCCTGTCACTTCTCAGAAATCGGTC	genomic DNA	*Asp*718 *Bam*HI
socs36E(II)-lacZ	GATAGGTACCGTTCTTGTCTGCGCTCGTTTC GGATGGATCCCTACGAACC GCGAAATCAACAC	genomic DNA	*Asp*718 *Bam*HI
socs36E(III)-lacZ	CTAAGGTACCGTGCGGCATGGAGTCGTGCATG GCTAGGATCCCGCATCAGTTAGTGCTCCCC	genomic DNA	*Asp*718 *Bam*HI
socs36E(IV)-lacZ	GCGCGGTACCGATGAAATCCAATCAAGTAGTGAC GTAAGCGGCCGCGCTGCTCGGCAGCATATC	genomic DNA	*Asp718* *Not*I
socs36E(I)-luc	GTTAGGTACCCTGTCTTAGGTGTTTACCAC CGTTAGATCTGTGCGAGTACGAGTATCTTTG	genomic DNA	*Asp*718 *Bgl*II
pUAST-STAT92E-GFP and mutant variants	GAGGTACCGAGCATGAGCTTGTGGAAGCGC GTTCTAGATTACTTGTACAGCTCGTCCATGCCGAGAGTG	*pBS(KS+)-STAT92E-GFP*	*Asp*718 *Xba*I

2.3.2 Polymerase chain reaction

Polymerase chain reaction (PCR) amplification of DNA fragments was used in this study for molecular cloning (see 2.3.1), site-directed mutagenesis (see 2.3.3) and the synthesis of templates for RNA *in situ* probes (see 2.3.7) and for double-stranded (ds) RNA used in RNAi experiments (see 2.4.4)

PCR reactions were generally set up in a volume of 50 µl and composed of:

1x PCR buffer (DNA polymerase-specific, supplied by manufactory)
0.25 mM dNTPs (Roche Diagnostics, Mannheim)
0.2 µM primers (MWG-Biotech AG, Ebersberg)

1U *Taq* (Fermentas, St. Leon-Rot) or 2.5U *PfuTurbo* DNA polymerase (Stratagene, Amsterdam, The Netherlands)

50ng (plasmid) to 1µg (genomic) DNA

General PCR cycling parameters were:

Pre-denaturation 2 min at 95°C

26 cycles of: denaturation 30 sec at 95°C

annealing 30 sec at appropriate temperature for the used pair of primers

synthesis 1 min per kb for *Taq* or 2 min per kb for *PfuTurbo* at 72°C

10 min to complete synthesis at 72°C

Primer annealing temperature was calculated from: $T_{annealing} = T_{melting} - 5°C$

All primers used in PCR reactions are shown in Table 2-3 (for primers used in molecular cloning see Table 2-2):

Table 2-3: Primers used for PCR.

Primer name	**Primer sequence**
Primers for synthesis of templates for RNA *in situ* probes and for dsRNA used in RNAi experiments (T7 promoter marked red):	
trh-F	GATTTCAGTGCCGACTCTCTG
T7trh-R	GAATTAATACGACTCACTATAGGGAGACTGATCCGTCTTGATGATGGT
STAT92E-F	TAATACGACTCACTATAGGGAGAAGCTGCTTGCCCAAAACTA
STAT92E-R	TAATACGACTCACTATAGGGAGCAGCTGAGAACCGATTAGCC
T7DOME-F	GAATTAATACGACTCACTATAGGGAGACCAGCTGCCTGACAAGCACC
DOMET7-R	GAATTAATACGACTCACTATAGGGAGACTGGACCCAGGCCCAATCCC
SOCT7-F (*socs36e*)	GAATTAATACGACTCACTATAGGGAGACCGCATCCACATCCGTGTCCAC
SOCT7-R (*socs36e*)	GAATTAATACGACTCACTATAGGGAGAAACGTGCCCTCCGGCTTG
SamS-F	TAATACGACTCACTATAGGGAGCGCTCAGATGTGTTCTGGTG
SamS-R	TAATACGACTCACTATAGGGAGATGGAGAGAGGCTCAGCAAG
PRMT1-F	TAATACGACTCACTATAGGGAGGGACAAGTGGCTGAAGAAGG
PRMT1-R	TAATACGACTCACTATAGGGAGGAAGTCCAGGTCACGGTTGT
CG3675-F	TAATACGACTCACTATAGGGAGATCTTGGATTCTGGCACGAT
CG3675-R	TAATACGACTCACTATAGGGAGGATCGGAGAGCACAAAGGAC
CG5358-F	TAATACGACTCACTATAGGGAGCCACGTGCTCAAGGAGTACA
CG5358-R	TAATACGACTCACTATAGGGAGCTTCCCCGGTATCACAGAGA
CG6563-F	TAATACGACTCACTATAGGGAGCCATCCATTCGGAAACTATGA

Primer name	Primer sequence
CG6563-R	TAATACGACTCACTATAGGGAGTCAGCAATCCGCTGTTGTAG
CG9882-F	TAATACGACTCACTATAGGGAGCGGCGATGACTACGACTACC
CG9882-R	TAATACGACTCACTATAGGGAGGCAATCGTTTTCAGGCTGTT
CG9927-F	TAATACGACTCACTATAGGGAGAACCAAAAATTGCCCTTCCT
CG9927-R	TAATACGACTCACTATAGGGAGCCATCCATTCGGAAACTATGA
CG9929-F	TAATACGACTCACTATAGGGAGAGGAGGTGATCTTTGCCAGA
CG9929-R	TAATACGACTCACTATAGGGAGCATCCCAAGCTCTCCGATTA
CG10061-F	TAATACGACTCACTATAGGGAGCGGCGTCTAAACAAAACTGC
CG10061-R	TAATACGACTCACTATAGGGAGGTTGAGATCGTCGAGCTGTG
CG16840-F	TAATACGACTCACTATAGGGAGTGCGATATTGGGGAATAAGG
CG16840-R	TAATACGACTCACTATAGGGAGCTTGCGGATTCAGCTGTGTA
CG32152(2)-F	TAATACGACTCACTATAGGGAGTAGTGTGGCAGAGCGAAAGA
CG32152(2)-R	TAATACGACTCACTATAGGGAGTCATGACCGTGATCACACCT
Primers for sequencing:	
STAT-S1	CAGGGCATAGGACCTATCCAC
STAT-S2	CGCGCAGAAGAACCTGGT
STAT-S3	CCGACCGCGTTACCTGGG
STAT-S4	CCGTTTCTCCGACAGCGAG
STAT-S5	CTCCACCGAGCTCGCTG
STAT-S6	CTCCGCAAATGCATTGTCCC
STAT-S7	CAGCTGAGAACCGATTAGCC
STAT-S8	GGGTGTGACCATACCGAGGG
Primers for site-directed mutagenesis (mutations introduced in STAT92E-GFP are shown in red):	
M647H-F	CGAAAATGGACTGGTCACCCACCTAGCGCCATGGACTGC
M647H-R	GCAGTCCATGGCGCTAGGTGGGTGACCAGTCCATTTTCG
Y711F-F (Y704F)	CTAGATCCTGTGACCGGTTTCGTGAAGAGCACATTACATG
Y711F-R (Y704F)	CATGTAATGTGCTCTTCACGAAACCGGTCACAGGATCTAG
STATR30A-F	GGAGAAGGGTATGCTCGAGCTGGCCCTCTGCTTGGCACCC
STATR30A-R	GGGTGCCAAGCAGAGGGCCAGCTCGAGCATACCCTTCTCC
STATR30E-F	GGAGAAGGGTATGCTCGAGCTGGAGCTCTGCTTGGCACCC
STATR30E-R	GGGTGCCAAGCAGAGCTCCAGCTCGAGCATACCCTTCTCC
STATR30K-F	GGAGAAGGGTATGCTCGAGCTGAAGCTCTGCTTGGCACCC
STATR30K-R	GGGTGCCAAGCAGAGTTCCAGCTCGAGCATACCCTTCTCC

2.3.3 Site-directed mutagenesis

Point mutations in pBS-STAT92E-GFP were generated using the QuickChange *in vitro* mutagenesis technique (Stratagene, Amsterdam, The Netherlands). Two or three successive rounds of mutagenesis were performed to generate double or triple mutations, respectively. All constructs were sequenced before they were sub-cloned into the inducible *Drosophila* germline transformation vector pUAST (Brand and Perrimon, 1993).

To generate point mutations at positions R30, M647 and Y704 of STAT92E-GFP, specific primers were designed carrying nucleotide substitutions (see Table 2-3). These point mutations converted the triplets CGC into GCC, GGC, GAG for R30A, R30E, R30K substitutions, ATG into CAC for M647H substitution and TAT into TTC for Y704F substitution, respectively. Following 7-10 cycles of PCR amplification (see 2.3.2) of pBS-STAT92E-GFP by *PfuTurbo* (Stratagene, Amsterdam, The Netherlands) using those primers, the resulting mixture containing the template and the newly synthesized plasmid was treated with *Dpn*I (Stratagene, Amsterdam, The Netherlands), a restriction endonuclease recognizing a 4 bp methylated DNA site. *Dpn*I digestion destroyed the methylated parental template. The newly synthesized mutated plasmid was transformed into bacteria. The resulting clones were analyzed, and *stat92E* ORFs carrying the desired mutations were re-cloned into the pUAST vector (Brand and Perrimon, 1993).

2.3.4 Endonuclease restriction

For molecular cloning or analysis of generated constructs restrictions were normally carried out for 1h at 37°C in 20 - 30 µl volume using 1 - 10U of the designated restriction enzyme and the respective buffer (Fermentas, St. Leon-Rot or New England Biolabs, Frankfurt a. M.). Restriction and PCR products were analyzed by electrophoresis on 0.8 - 1.5% agarose gels in 0.5x TBE buffer. The DNA fragments were stained with ethidium bromide and visualized with an UV-transilluminator (Raytest, Straubenhardt) at 366nm. The GeneRuler DNA Ladder Mix (Fermentas, St. Leon-Rot) was used to control fragment sizes.

DNA fragments were purified by either ethanol precipitation (salting agent 500 mM NaCl), air-dried and dissolved in EB buffer (Qiagen, Hilden), or the desired band was excised from the gel and purified using the Gel Extraction Kit (Qiagen, Hilden).

2.3.5 Ligation

For ligation cut and purified fragments were mixed with similar cut vector DNA (see 2.3.4) in a ratio of approximately 4:1. Ligation reactions were carried out in a total volume of 20 µl o/n at 16°C in 1x ligation buffer using 1U of T4 DNA ligase (Roche Diagnostics, Mannheim).

2.3.6 Heat shock transformation of bacterial cells

Plasmids were introduced into chemical competent *E. coli* XL1-Blue cells by heat shock transformation. Plasmids or ligation samples were mixed with 100 µl competent cells and incubated 45 min on ice. A 45 - 55 sec heat shock at 42°C followed. After two min chilling on ice cells recovered in 900 µl LB^- medium for 1h at 37°C, were then spread on agar plates supplemented with the appropriate antibiotic and grown o/n at 37°C.

Colonies containing the desired plasmid DNA were used to inoculate in 5 ml LB^+ medium and grown o/n at 37°C. 1 ml of the bacterial suspension was used for analytical DNA preparation by the QIAprep Spin Miniprep Kit (Qiagen, Hilden). Presence of the cloned DNA fragments was tested by analytical restriction digests (see 2.3.4) and, if necessary, confirmed by DNA sequencing. The bacterial colonies containing the desired DNA constructs were used to inoculate in 100 - 150 ml LB^+ medium and grown o/n at 37°C. Plasmid DNA preparation was performed using the Plasmid Maxiprep Kit (Qiagen, Hilden).

Table 2-4: Plasmids used.

Construct	Remarks	Reference
pUC18-Act-Gal4	constitutively expresses Gal4	
pUAST	vector for Gal4 mediated transgene expression (see 2.7.4)	Brand and Perrimon, 1993
pAct-Renilla	constitutively expresses *Renilla* luciferase	Müller *et al.*, 2005; Karsten *et al.*, 2006
pAct-Upd-GFP	constitutively expresses Upd-GFP	Müller *et al.*, 2005
pAct-Hop^{Tuml}	constitutively expresses Hop^{Tuml}	Müller *et al.*, 2005
2x*Draf*STATwt-TATA-luc	JAK/STAT-dependent luciferase reporter construct containing 2 STAT92E binding sites of the *Draf* promoter	Kwon *et al.*, 2000
6x2xDrafLuc	multimerized form of 2x*Draf*STATwt-TATA-luc	Müller *et al.*, 2005
pW8ßE	vector for P-element-mediated germ line transformation, includes *lacZ* ORF	modified after Klemenz *et al.*, 1987

2.3.7 RNA probe labeling for *in situ* hybridization

For RNA *in situ* hybridization experiments (see 2.8.1.2) anti-sense RNA probes were labeled with DIG-coupled UTPs using the DIG-Labeling Kit (Roche Diagnostics, Mannheim). Genomic DNA or plasmids containing the corresponding cDNA were used as template (see Table 2-5). The labeling reaction was performed according to the manufacturer´s instructions. The labeled probe was subsequently mixed with 10 µl of 20 mg/ml non-specific tRNA (Sigma-Aldrich, Taufkirchen) in a final volume of 200 µl (adjusted with DEPC-treated H_2O). The RNA was precipitated by ethanol (salting agent 400 mM LiCl) for at least 2h at –80°C, subsequently centrifuged at 13000 rpm for 15 min, washed twice with 70% ethanol, air-dried and dissolved in 100 µl of DEPC-treated H_2O for 30 min at 37°C.

The efficiency of labeling was tested by dot-blot hybridizations. 1 µl of serial dilutions of the probes (1:10, 1:100, 1:1000) and control RNA (1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl) were spotted onto a nylon membrane (Amersham Biosciences, Freiburg). When fully dried, the nucleic acids were cross-linked to the membrane by UV-light for two min. The membrane was afterwards washed two times for five min with PBS, blocked with PBS supplemented with 10 µg/ml BSA and 5% sheep serum for 30 min and hybridized with anti-DIG alkaline phosphatase (AP)-conjugated antibody (1:5000 dilution, Roche Diagnostics, Mannheim) for 30 min at RT to detect incorporated DIG-coupled UTP in the synthesized probes. The membrane was then washed three times for ten min with PBS to remove unbound antibodies. After two washing steps with AP-buffer of ten min each, colorimetric detection of the alkaline phosphatase was performed using NBT/BCIP solution (20 µl of stock solution (Roche Diagnostics, Mannheim) per 1 ml AP-buffer). This reaction was stopped by several washes with PBS. The intensity of the experimental spots was compared with the control, and the approximate concentration of the labeled probe was estimated (suggested working concentration 1 ng/ml).

For DEPC-treatment 1 ml DEPC (Sigma-Aldrich, Taufkirchen) per 1l H_2O was constantly mixed for 1h at 37°C or o/n at RT and autoclaved afterwards.

Table 2-5: Scheme for RNA probe preparation.

Probe	Template	Restriction enzyme / primers	Polymerase
socs36E	*pOT2A-socs36E*	*Eco*RI	SP6
lacZ	*pBS-KS-ßgal*	*Not*I	T3
trh	genomic DNA	trh-F, T7trh-R (see Table 2-3 for primer sequences)	T7

2.3.8 Isolation of genomic DNA from flies

For genomic DNA isolation approximately 50 flies per sample were frozen and ground in 400 µl DNA extraction buffer using a plastic pestle. Additional 400 µl DNA extraction buffer can help to rinse the pestle. The homogenate was incubated for 30 min at 65°C. After the addition of 120 µl 8 M K-acetate the sample was incubated for 30 min on ice. After centrifugation at 13000 rpm for five min the supernatant was transferred into a fresh tube. The DNA was purified by ethanol precipitation. After centrifugation the pellet was washed with 70% ethanol, dried and resuspended in 400 µl TE buffer. RNAs were removed by RNAse treatment (final concentration 2 µg/ml) for 30 min at 37°C. The DNA was subsequently purified by phenol/chloroform extraction followed by ethanol precipitation. Finally, the DNA was dissolved in 50 µl TE buffer with an approximate DNA concentration of 1 µg/µl.

2.4 *Drosophila* cell culture & cell culture based assays

2.4.1 Maintenance of cultured cells

S2 (Schneider, 1972), Kc_{167} (Echalier and Ohanessian, 1969) and S2R+ (Yanagawa *et al.*, 1998) cultured *Drosophila* cells were grown in 75cm^2 flasks (BD Biosciences, Heidelberg) containing 10 ml Schneider's *Drosophila* medium (Gibco Invitrogen, Karlsruhe) supplemented with 10% fetal calf serum (Sigma-Aldrich, Taufkirchen) and penicillin/streptomycin (0.1 mg/ml, PAA Laboratories, Pasching, Austria GmbH) at 25°C.

2.4.2 Transfections

JAK/STAT pathway components were ectopically expressed in cultured cells for functional analysis of this signal cascade. To do so, cells were seeded three to six h before transfection. In general, 3x10^4 cells per well of a 96-well plate, 5x10^5 per well of a 6-well plate and 1x10^6 (5x10^6) cells onto 60mm (100mm) dishes were seeded.

Transfections were performed using the Effectene Transfection Reagent (Qiagen, Hilden) according to the manufacturer´s instructions except using 11 µl instead of 22 µl Effectene Reagent per 1 µg DNA.

Methylation in cultured cells was inhibited to assess its role for JAK/STAT signaling. For this purpose, cultured cells were treated with 5´-deoxy-5´(methylthio)adenosine (MTA, Sigma-Aldrich, Taufkirchen), which was dissolved in Schneider's *Drosophila* medium (Gibco

Invitrogen, Karlsruhe). MTA was added 6h after transfection in the final concentration of 0.1 or 0.5 mM and was incubated with the cells until lysis.

For inhibition of protein tyrosine phosphatases for EMSA analysis (see 2.5.3) sodium-ortho-vanadate (Sigma-Aldrich, Taufkirchen) was used. 50 µl sodium-ortho-vanadate (1M) and 55 µl H_2O_2 (31% stock) were incubated in 1 ml Schneider's *Drosophila* medium (Gibco Invitrogen, Karlsruhe) for five min at RT. This mixture was added to the transfected cells in a final concentration of 100 µM sodium-ortho-vanadate and 1 µM H_2O_2 for 45 min before lysis.

2.4.2.1 Transfections for dual luciferase reporter assays

For dual luciferase reporter assays cultured cells were seeded in 96-well plates. Transfections were performed as described in 2.4.2. Transfection mixtures (200 ng DNA per well) were set up in replicates. 65 ng reporter construct, 35 ng *pAct-Gal4* and 5 ng *pAct-Renilla* was transfected per well. Additionally, to test the effect of STAT92E-GFP variants, 55 ng *pUAST-STAT92E-GFP* was co-transfected with 40 ng *pUAST-Hop$^{(Tuml)}$* or *Act-Hop$^{(Tuml)}$*. For mock or non-stimulation transfections *pUAST* and *pAct* constructs were substituted for equal amounts of each of the respective empty *pUAST*- and *pAct* constructs. For transfection in replicates plasmid mixtures were prepared in the respective multiple amounts of DNA specified above. Before addition to the cells, the transfection mixtures were split to transfect 200 ng DNA in total per well. Cells were lysed 72h after transfection according to the Dual-Luciferase Assay Kit (Promega, Mannheim). Lysates were then used in a dual luciferase assay for measurement of luciferase activities (see 2.4.3.2).

2.4.2.2 Transfections for EMSA and Western blotting experiments

For EMSA and Western Blotting experiments cultured cells were seeded in 60mm (100mm) tissue culture dishes. Transfections were performed as described in 2.4.2 with 500 (750) ng *pAct-Gal4* and 2 ng *pUAST-luc* as normalization control. To express STAT92E-GFP variants, 500 (750) ng *pUAST-STAT92E-GFP* was co-transfected with 500 ng *pUAST-Hop$^{(Tuml)}$* or *Act-Hop$^{(Tuml)}$*. For mock or non-stimulation transfections *pUAST* and *pAct* constructs were substituted for equal amounts of each of the respective empty *pUAST* and *pAct* constructs. Cells were lysed 72h after transfection (see 2.5.1 for immunoprecipitation and Western blotting or 2.5.3 for EMSA analysis).

2.4.2.3 Transfections for immuno-staining and sub-cellular localization assays

For immuno-staining and sub-cellular localization experiments cultured cells were seeded in 6-well plates on glass coverslips. Transfections were performed as described in 2.4.2 with 300 ng *pAct-Gal4*. To express *STAT92E-GFP* variants, 500 ng *pUAST-STAT92E-GFP* was co-transfected with 200 ng *pUAST-Hop*$^{(Tuml)}$ or *Act-Hop*$^{(Tuml)}$. For mock or non-stimulation transfections *pUAST* and *pAct* constructs were substituted for equal amounts of each of the respective empty *pUAST* and *pAct* constructs. Cells were fixed and mounted 72h after transfection (see 2.8.2).

2.4.3 Luciferase assays

Firefly and *Renilla* luciferase activities were measured on a Wallac Victor Light 1420 luminescence counter (PerkinElmer, Rodgau-Jügesheim) using the Dual-Luciferase Reporter Assay (Promega, Mannheim).

2.4.3.1 Firefly luciferase activity measurement

Firefly luciferase activity was used for normalization of the relative transfection efficiency of cells prepared for EMSA experiments (see 2.5.3). Firefly luciferase was consitutively expressed in cultured cells (see 2.4.2.2), and its activity in cell lysates was determined according to the manufacturer´s instructions. Measurements were performed in non-transparent 96-well reading plates. 25 µl LAR-II solution (luciferase assay reagent, Promega, Mannheim) was injected into each well containing 5-10 µl of the lysate (see 2.4.2.2). After a delay of two sec, the luminescent signal was measured for ten sec.

2.4.3.2 Dual luciferase reporter assay

2x*Draf*STATwt-TATA-luc (Kwon *et al.*, 2000) or *6x2xDrafLuc* (Müller *et al.*, 2005) reporter activation dependent on JAK/STAT activity results in firefly luciferase expression. *Renilla* luciferase was ubiquitously expressed using the *actin5.1* promoter to assay for transfection efficiency and as a control suitable for normalization. Both firefly and *Renilla* luciferase activities were analyzed using the Dual-Luciferase Reporter Assay (Promega, Mannheim). 20 µl of the lysates of transfected cells (see 2.4.2.1) were used for measuring. 25 µl LAR-II solution (Promega, Mannheim) containing the substrate for the firefly luciferase were added

to the cell lysates. After a delay of three sec, measurement of the luminescent signal was performed for ten sec. Afterwards, the reaction was quenched, and the *Renilla* luciferase reaction was started simultaneously by addition of 25 µl Stop&Glo solution (Promega, Mannheim). After another delay of three sec, the luminescent signal of *Renilla* luciferase was measured for ten sec. Relative reporter activity was calculated from the ratio of firefly to *Renilla* luciferase activity. Reporter activation values in experimental samples were normalized to mock transfected cells.

2.4.4 RNA interference

The dual luciferase reporter system (see 2.4.3.2) was also used in RNAi assays to identify genes that interact with the JAK/STAT pathway. Candidate gene expression was knocked down in cultured cells by the addition of sequence-specific dsRNA. The effect of the resultant reduced candidate gene expression on JAK/STAT signaling was detected using the dual luciferase reporter assay. dsRNA targeting candidate genes was generated from DNA fragments covering 400 - 600bp of the respective gene. These fragments were amplified by PCR on genomic DNA (see 2.3.2) using gene-specific primers introducing a T7 RNA polymerase promoter (see Table 2-3). The gene specific region of the primers was directed against single exons of the candidate genes. One-tenth of the PCR sample was used for subsequent generation of dsRNA in an *in vitro* transcription reaction using T7 RNA polymerase:

PCR template
1x transcription buffer (Fermentas, St. Leon-Rot)
10 mM DTT (Sigma-Aldrich, Taufkirchen)
1 mM NTPs (Roche Diagnostics, Mannheim)
60U RNAse inhibitor (Fermentas, St. Leon-Rot)
10U T7 RNA polymerase (Fermentas, St. Leon-Rot)

This reaction was carried out for at least 2h at 37°C. Afterwards, DNA was degraded by DNAse treatment (15U, Fermentas, St. Leon-Rot) for 15 min at 37°C. The RNA was then purified by ethanol precipitation, air-dried and dissolved in RNAse-free H_2O. Formation of dsRNA was achieved by heating the RNA for one min to 95°C and subsequent cooling down to RT.

For RNAi interaction assays 1.5 µg dsRNA / $1x10^5$ cells were added to cultured cells 2h after transfection to specifically knock down candidate gene expression. The cells were incubated with dsRNA until lysis for the dual luciferase reporter assay (see also 2.4.2.1).

2.5 Protein biochemistry

2.5.1 Immunoprecipitation

Immunoprecipitation (IP) and Western Blotting experiments were performed to monitor post-translational modifications of STAT92E-GFP variants, which were expressed in cultured cells (see 2.4.2.2). To obtain crude cell lysates, cells were rinsed several times in cell rinse buffer, transferred into 1 ml cell rinse buffer and centrifuged at 13000 rpm for two min at RT. The cell pellet was then resuspended in 400 µl cell resuspension buffer and subjected to three cycles of freezing in liquid nitrogen and thawing. Cellular debris was removed by centrifugation at 13000 rpm for five min at 4°C. The supernatent was stored at -80°C.

For IP RIPA buffer was added to the crude cell lysates to a total volume of 470 µl. Lysates were then mixed with anti-GFP antibody (1:500, Abcam, Cambridge, UK) and rocked for 3h at 4°C. 50 µl of 50% Protein-A agarose beads (Calbiochem, Bad Soden) were washed in RIPA buffer three times for five min at 4°C, added to the lysates and incubated on a rocking platform o/n at 4°C. The beads were washed in RIPA buffer five times for 20 min, centrifuged, mixed with 15 µl of 2x Laemmli buffer (Laemmli, 1970) and boiled for five min. The eluted proteins were resolved by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and analyzed by Western blotting (see 2.5.2).

2.5.2 SDS-Polyacrylamid Gel Electrophoresis & Western Blotting

Discontinous gels were composed of 8% polyacrylamide for the separating gel and 5% polyacrylamide for the stacking gel. Electrophoresis was performed in 1x SDS-running buffer at 35mA constant current. Pre-stained SDS-PAGE Standards (New England Biolabs, Frankfurt a. M.) were used as molecular weight control.

For Western Blotting the unstained gel was rinsed with transfer buffer immediately after electrophoresis and put onto a piece of nitrocellulose membrane (Schleicher & Schuell, Dassel) moistened with transfer buffer. The protein transfer apparatus was assembled in a way that the membrane was oriented to the anode, while the gel faced the cathode to allow the

transfer of the negatively charged SDS-coated proteins onto the membrane. Western Blotting was carried out at 150mA per gel for 1h at 4°C. Afterwards, the membrane was rinsed in PBS and subsequently blocked in PBS with 10 mg/ml BSA for 1h at RT or o/n at 4°C. Primary antibody incubation was carried out o/n at 4°C (for an overview of the antibodies, see Table 2-6). Unbound antibody was removed by washing with PBS four times for ten min. The membrane was then incubated with species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (Perbio Science, Bonn, see Table 2-6) in PBS with 10 mg/ml BSA for 1 - 2h at RT. Subsequent washing steps were performed similar to the ones after primary antibody incubation. The coupled peroxidase was incubated with the SuperSignal West Pico Chemiluminescent Substrate (Perbio Science, Bonn) for five min at RT. Luminescence was detected either on a Fujifilm Luminescent Image Analyser LAS-1000 CH or using BioMax XAR films (Kodak, Stuttgart).

Table 2-6: Antibodies used.

Primary antibodies		
rabbit polyclonal antibody to GFP	1:5000 (Western blotting) 1:500 (IP)	Abcam ab290-50
Mouse monoclonal (7E6) to Arginine (mono- and di-methyl)	1:500 (Western blotting) 1:100 (IP)	Abcam ab412-200
Secondary antibodies		
Goat anti-mouse HRP conjugated	1:8000	Pierce #1858412
Goat anti-rabbit HRP conjugated	1:8000	Pierce #1858415

2.5.3 Electro Mobility Shift Assay

Electro Mobility Shift Assays (EMSA) were used to test for the ability of STAT92E-GFP variants to bind radio-labeled oligonucleotides containing a STAT92E consensus recognition site (Yan *et al.*, 1996).

DNA probe labeling and cold oligonucleotides synthesis for EMSA

Single-stranded DNA oligonucleotides with partly complementary sequences were designed containing a STAT92E consensus or a mutated recognition site (for sequences see Table 2-7). 5pmole of each oligonucleotide were mixed with Restriction Buffer 2 (New England Biolabs, Frankfurt a. M.) in a final volume of 100 µl, denatured for ten min at 95°C and let cool down

to RT to allow the annealing of the single-stranded oligonucleotides. The resulting dsDNA fragments with the calculated concentration of 5 µM contained 3'-overhangs, which were filled by Klenow with dNTPs containing ^{32}P-dCTP. The composition of the labeling reaction in a volume of 50 µl was as followed:

1x Restriction buffer 2 (New England Biolabs, Frankfurt a. M.)
0.5 µM annealed oligonucleotides
0.4 mM each dATP, dTTP and dGTPs (Roche Diagnostics, Mannheim)
0.33 µM ^{32}P-dCTP (Hartmann Analytic, Braunschweig)
2U Klenow fragment (Roche Diagnostics, Mannheim)

The reaction was incubated for 20 min at 37°C.

The synthesized probe was purified using the Nucleotide Removal Kit (Qiagen, Hilden) according to the manufacturer´s instructions. When eluted in 100 µl EB buffer, the calculated concentration of the probe was 250 nM. The labeling efficiency was measured on Bioscan QC-4000 XER. Generally, 1 µl of the probe gave 100000 – 700000 cpm (counts per min). 10000 cpm are sufficient for one EMSA reaction.
Synthesis of cold oligonucleotides was performed similarly, except that the labeling reaction was carried out in a volume of 100 µl, the annealed oligonucleotides were added in 10x higher concentration, and ^{32}P-dCTP was replaced with dCTP with the final concentration of 0.5 mM for each dNTP. When eluted in 100 µl EB buffer, the calculated concentration of cold oligonucleotides was 2.5 µM.

Table 2-7: Sequence of oligonucleotides used for STAT92E probe labeling. Nucleotides essential for STAT92E binding are depicted in red.

Oligonucleotide	Probe	Sequence
wtSTAT-top	WT: oligonucleotide containing a STAT92E recognition site	GGATTTTTCCCGGAAATG
wtSTAT-bottom		GACCATTTCCGGGAAAAA
mutSTAT-top	mut: oligonucleotide containing a mutated STAT92 recognition site	GGATTTTTGCCGCAAATG
mutSTAT-bottom		GACCATTTGCGGCAAAAA

EMSA

Cultured cells were incubated five min on ice in lysis buffer three days after transfection (see 2.4.2.2). Following centrifugation (13000 rpm, five min at 4°C) to remove cell debris, the supernatant was used for EMSA and Western Blotting analysis to normalize for transfection efficiency. Radio-labeled or unlabeled STAT92E probes containing the consensus STAT92E recognition sequence (Yan *et al.*, 1996) or a mutated recognition sequence were generated (see above) to detect the DNA binding activity of STAT92E-GFP variants. 2 µl (16.67 nM) STAT92E probe and protein extract normalized for firefly luciferase activity (see 2.4.3.1) were allowed to bind for 30 min at RT in binding buffer. For supershift experiments anti–GFP antibody (1:3000, Abcam, Cambridge, UK) was added to the binding reaction. For cold oligonucleotide competition assays unlabeled STAT92E probes were added to the binding reaction in a 10x, 50x and 100x excess. Complexes were resolved by native polyacrylamide gel electophoresis in a 5% continous gel, 0.5x TBE before drying and autoradiography.

2.6 Computional analysis

For identification of potential protein arginine methyltransferases (PRMT), Basic Local Alignment Search Tool (BLAST) searches of the *Drosophila* proteome were performed (blastp at http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al.*, 1990).

Protein alignments were performed using the clustal alignment algorithm of MegAlign software (DNASTAR Inc.; Higgins and Sharp, 1989).

Identification of conserved domains in candidate proteins was performed using InterProScan (http://www.ebi.ac.uk/InterProScan/; Zdobnov and Apweiler, 2001).

Sequence identities of proteins used for BLAST searches and alignments: STAT5A (*Homo sapiens*): NP_003143, STAT5A (*Mus musculus*): NP_035618, STAT5B (*Gallus gallus*): NP_990110, STAT5.1 (*Danio rerio*): NP_919368, STAT5 (*Xenopus laevis*): AAK94906, STAT (*Spodoptera frugiperda*): AAL37477, STAT92E (*Drosophila melanogaster*): NP_996242, STAT (*Anopheles gambiae*): CAA09070, STAT (*Caenorhabditis elegans*): AAY18583, PRMT1 (*Homo sapiens*): AAH19268. Potential PRMTs in *Drosophila melanogaster*: CG6554/Dart1: NP_650017, CG3675/Dart2: NP_608821, CG6553/Dart3: NP_650434, CG5358/Dart4: NP_649963, CG9927/Dart6: NP_650322, CG9882/Dart7: NP_611753, CG16840/Dart8: NP_609478, CG9929/Dart9: NP_650321, CG32152: NP_730116.

2.7 Fly work & genetics

2.7.1 Fly stocks

The following fly stocks were used:

OreR (*Oregon R*, *Drosophila* wild type strain), w^{1118}, *y w P{w*$^{+}$*, GMR-updΔ3´}/FM7, P{w*$^{+}$*, Ubp-GFP}* (Bach *et al.*, 2003), *w; P{w*$^{+}$*, ey-Gal4}* (Halder *et al.*, 1995), *w; P{w*$^{+}$*, Nullo-Gal4}* (Kunwar *et al.*, 2003, kindly provided by W. Gehring), *y w; P{w*$^{+}$*, UAS-GFP}* (Karsten *et al.*, 2006), *w; P{w*$^{+}$*, UAS-domeΔCYT}/TM3* (Brown *et al.*, 2001), *w; P{w*$^{+}$*, UAS-Hop*^{Tuml}*}* (Harrison *et al.*, 1995), *M(2)21AB*^{1}*/CyO, P{ry*$^{+t7.2}$*=ftz/lacZ}USC1* (Bloomington stock #6305), *y*^{1}*; P{y*$^{+mDint2}$*w(BR.E.BR)=SUPor-P}Art1*^{KG09631}*, ry*^{506} (Bloomington stock #15202).

2.7.2 Fly handling and feeding

The handling and feeding of *Drosophila melanogaster* was performed according to Ashburner (Ashburner, 1989). All embryos, larvae and flies were grown at 25°C on standard cornmeal/agar food, unless otherwise specified.

2.7.3 Genetic interaction assay

To test for genetic interaction of JAK/STAT signaling and components of the methylation machinery, *y w P{w*$^{+}$*, GMR-updΔ3´}/FM7, P{w*$^{+}$*, Ubp-GFP}* virgins were crossed to males of following genotypes: *M(2)21AB*^{1}*/CyO, P{ry*$^{+t7.2}$*=ftz/lacZ}USC1* and *y*^{1}*; P{y*$^{+mDint2}$*w(BR.E.BR)=SUPor-P}Art1*^{KG09631}*, ry*^{506}. As negative control, *y w P{w*$^{+}$*, GMR-updΔ3´}/FM7, P{w*$^{+}$*, Ubp-GFP}* virgins were crossed to OreR males. Crosses were set up in parallel on the same batch of food. For *GMR-updΔ3´* interactions any deviation from the size of the overgrown *GMR-updΔ3´/+* adult eye was scored as potential JAK/STAT pathway interaction.

2.7.4 Ectopic expression using the Gal4/UAS system

The Gal4/UAS system is based on the yeast transcription factor Gal4 and its recognition site UAS (upstream activating sequence) (Brand and Perrimon, 1993). Using this system, any cloned gene can be ectopically expressed in defined tissues. A driver line carries either a tissue-specific or inducible enhancer sequence upstream of the Gal4 gene. An effector line is

composed of an upstream UAS promoter and a downstream gene of interest. When crossed to the driver, the target transgene is expressed in the same tissue- and stage specific pattern as the transcription factor Gal4.

In this study, the driver *ey-Gal4* (Halder *et al.*, 1995) was used for expression in the eye imaginal disc during *Drosophila* eye development. For expression in all somatic tissues during *Drosophila* embryogenesis at blastoderm stage the *nullo-Gal4* driver (Kunwar *et al.*, 2003) was used.

2.7.5 Preparing DNA for subsequent injection into *Drosophila* embryos

DNA constructs destined to be injected in *Drosophila* embryos (see 2.3) were extracted with the MidiPrep DNA purification Kit (Qiagen, Hilden). Subsequently, they were additionally purified by phenol/chloroform extraction and precipitated with ethanol (salting agent 300 mM Na-acetate, pH 5.5). Following centrifugation the resulting pellet was washed with 70% ethanol, air-dried and dissolved in TE buffer for five min at 60°C.

12 µg of the purified DNA construct was mixed with 4 µg "helper DNA" coding a transposase and co-precipitated by ethanol (salting agent 300 mM Na-acetate, pH 5.5). The precipitate was centrifuged, washed twice with 70% ethanol, air-dried and dissolved in 20 µl H_2O for five min at 60°C. DNA concentration was adjusted to 400 ng/µl.

2.7.6 P-element-mediated generation of transgenic flies

Transgenic flies were generated as described by Rubin and Spradling, 1982. Purified DNA constructs (see 2.7.5) intended to be integrated into the fly genome were injected into preblastoderm embryos of the *Drosophila* w^- strain. Each hatched fly was crossed to w^- mutant flies. Integration of the transgene into the genome could be detected by a reversion of the mutant phenotype to red eye color by introduction of the *mini-white* gene on the transgene construct.

Table 2-8: Transgenic lines generated.

Genotype	**Eye phenotype** (upon over-expression using *ey-Gal4*) **and remarks**
w; P{w$^+$, UAS-STAT92E-GFP}1a.2/(CyO)	no change
w; P{w$^+$, UAS-STAT92E^{Y704F}-GFP}1b.3/(TM3)	no change
w; P{w$^+$, UAS-STAT92E^{M647H}-GFP}1/(CyO)	reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{M647H}-GFP}2.1/(TM3, Sb)	slightly reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{M647H}-GFP}4.1/(TM3, Sb)	slightly reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{M647H}-GFP}6.0/(CyO)	slightly reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{M647H}-GFP}11.0/(TM3, Sb)	slightly reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{R30A}-GFP}1.0.3/(TM3,Sb)	strongly reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{R30A}-GFP}2.0.1/FM6	varies from moderate to strong size reduction
w; P{w$^+$, UAS-STAT92E^{R30E}-GFP}3.III/(TM3)	varies from dramatically reduced size to complete loss, rough structure. Eyes are not equally affected.
w; P{w$^+$, UAS-STAT92E^{R30E}-GFP}2.1.3/(TM3)	reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{R30E}-GFP}3.1.II/(CyO)	slightly reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{R30K}-GFP}1.II/(CyO)	reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{R30A,M647H}-GFP}2.1.3/(TM3, Sb)	varies from strong size reduction to complete loss
w; P{w$^+$, UAS-STAT92E^{R30E,M647H}-GFP}1.2/(CyO)	varies from strong size reduction to complete loss
w; P{w$^+$, UAS-STAT92E^{R30E,M647H}-GFP}1.0.2/(CyO)	strongly reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{R30K,M647H}-GFP}2.1.2/(CyO)	reduced size and rough structure
w; P{w$^+$, UAS-STAT92E^{R30A,Y704F}-GFP}3.0.2/(CyO)	no change
w; P{w$^+$, UAS-STAT92E^{R30A,Y704F}-GFP}7.0.2/(CyO)	no change
w; P{w$^+$, socs36E(I)-lacZ}/(CyO)	transgenic flies carrying *lacZ* reporter constructs dependent on fragments of the *socs36E* genomic region containing 3 -5 potential STAT92E binding sites
w; P{w$^+$, socs36E(II)-lacZ}/(CyO)	
w; P{w$^+$, socs36E(III)-lacZ}/TM3	
w; P{w$^+$, socs36E(IV)-lacZ}/(CyO)	

2.8 Histology

2.8.1 *Drosophila* embryo manipulations

For embryo collections flies were kept on apple juice agar plates holding baker´s yeast as extra food. To collect embryos of all stages, flies were allowed to lay eggs for 20h at 25°C.

2.8.1.1 Embryo fixation

Embryos were washed off the apple juice agar plates with H_2O, cleansed of the yeast, transferred to a fine sieve and dechorionated in 50% DanKlorix bleach (Colgate-Palmolive, Hamburg) for five min. The dechorionated embryos were rinsed several times with H_2O, transferred to a glass vial and fixed in 1 ml RNA fixative and 6 ml heptane for 20 min with vigorous shaking. The lower phase containing the fixative was replaced with 6 ml of methanol. Afterwards, the vitelline membrane of the embryos was removed by vigorous shaking for one min. Devitellinated embryos sank to the bottom of the vial. The upper phase was discarded. Embryos were washed several times in methanol, collected in Eppendorf tubes and stored at -20°C.

2.8.1.2 Whole-mount RNA *in situ* hybridization

Gene expression can be analyzed by RNA *in situ* hybridization using DIG labeled RNA probes (see 2.3.7). This way, the expression pattern of genes can be detected. To do so, embryos stored in methanol (see 2.8.1.1) were re-hydrated in 50% methanol/PBS and afterwards post-fixed in 500 µl PBT and 500 µl RNA fixative for 20 min. After several washes with PBT, embryos were treated with Proteinase K (50 µg/ml, Sigma-Aldrich, Taufkirchen). This treatment was stopped after 90 sec by the addition of glycine (2 mg/ml). The embryos were then rinsed two times with PBT and post-fixed once again in 500 µl PBT and 500 µl RNA fixative for 20 min. Embryos were washed several times in PBT and afterwards equilibrated in a mixture of PBT/hybridization buffer (Hybe) B (1:1) and in Hybe B alone. Hybe B was replaced with Hybe, and embryos were pre-hybridized for 1h at 70°C. Afterwards, the embryos were hybridized with RNA *in situ* probes (see 2.3.7) in a minimal solution of Hybe (around 50 µl) o/n at 70°C. The next day 500 µl Hybe were added, replaced with Hybe B and washed two times for 15 min at 70°C with pre-warmed Hybe B. Then 500 µl PBT was added. After several rinses and washing steps of 15 and 20 min in PBT at RT,

incubation with anti-Dig AP-conjugated antibody (1:2000, Roche Diagnostics, Mannheim) for 1h followed. Embryos were rinsed several times in PBT, then washed for 20 and 30 min in PBT to remove unbound antibodies followed by two short washing steps in AP buffer. Colorimetric detection of the alkaline phosphatase was performed using NBT/BCIP solution (20 µl of stock solution (Roche Diagnostics, Mannheim) per 1 ml AP-buffer). The staining reaction was stopped by several rinses with PBT and a subsequent 20 min washing step.
The stained embryos were dehydrated in an ethanol dilution series (30%, 50%, 70%) and left in 70% ethanol o/n at 4°C. The next day embryos were dehydrated in 100% ethanol and mounted in a 1:1 mixture of Canada Balsam (Sigma-Aldrich, Taufkirchen) and methyl salicylate (Sigma-Aldrich, Taufkirchen). Mounted embryos were photographed using a Zeiss Axioskop2 MOT microscope.

2.8.1.3 Whole-mount antibody detection of proteins

To specifically detect protein expression in embryos, whole-mount antibody stainings were undertaken. To do so, embryos stored in methanol (see 2.8.1.1) were re-hydrated in methanol/PBS dilution series (4:1, 3:2, 2:3, 1:4). Unless otherwise specified, all procedures were performed at RT. The fully re-hydrated embryos were washed in PBT three times for 20 min, blocked in PBT with 2% sheep serum and, finally, incubated with anti-lacZ primary antibody (1:3000, Cappel, Durham, NC, USA) o/n at 4°C. The embryos were washed in PBT three times for five min, then two times in PBT with 2% sheep serum. Following incubation with preabsorbed species-specific biotin-conjugated secondary antibody (1:500 dilution, Vectastain ABC Elite Kit, Vector Laboratories, Peterborough, UK) in PBT with 2% serum for 2h at RT, embryos were washed four times for ten min in PBT.
For detection the embryos were incubated with a complex of avidin/biotinylated HRP (preformed for 30 min, Vector Laboratories, Peterborough, UK) for 30 - 90 min. The embryos were then washed thoroughly four times for 20 min. Colorimetric detection of the HRP enzyme activity was performed by addition of 30 µl diaminobenzidine tetrachloride (10 mg/ml stock) and 10 µl H_2O_2 (37% stock) to the embryos in 0.5 ml PBT resulting in brown staining. Coloring can be achieved very fast and should be monitored under a microscope. To stop the reaction, the embryos were washed several times in PBT. Finally, the embryos were dehydrated in ethanol dilution series (10%, 30%, 50%, 70%, 90%), washed twice in 100% ethanol, clarified in 0.5 ml methyl salicylate (Sigma-Aldrich, Taufkirchen) and mounted in

Canada balsam (Sigma-Aldrich, Taufkirchen). Embryos were imaged with Zeiss Axioskop2 MOT microscope.

2.8.2 Immuno-staining and sub-cellular localization of STAT92E-GFP variants

Transfected cells (see 2.4.2.3) were washed in PBS, fixed in PBS + 5% formaldehyde three to four days after transfection and then mounted in VectaShield Mounting Medium with DAPI (H-1200, Vector Laboratories, Peterborough, UK). The cells were analyzed for the sub-cellular localization of STAT92E-GFP variants using a Zeiss Axioskop microscope, Axiocam and Openlab software (Improvision, Tübingen).

For immuno-stainings cells were fixed as described above. Afterwards, they were incubated with anti-phospho-STAT92E antibody (1:200, Cell Signaling Technologies, Danvers, MA, USA) for 1h in PBT with 2% sheep serum. After washing with PBS, incubation for 1h with Cy3-conjugated secondary antibody (1:200, Jackson Immuno Research Labs, Cambridgeshire, UK) followed. After another washing step with PBS, cells were mounted in VectaShield Mounting Medium (Vector Laboratories, Peterborough, UK). Images were captured on a Leica TCS SP2 confocal microscope using sequential scans.

3 Results

3.1 Tools for the analysis of *Drosophila* JAK/STAT pathway activity

In order to analyze and visualize *Drosophila* JAK/STAT pathway activity in general and the function of STAT92E variants in particular, cell culture and *in vivo* assays have been established.

3.1.1 Transcriptional control of *socs36E*

SOCS proteins act as negative regulators of the JAK/STAT pathway, and several SOCS genes are transcriptional targets of this signaling cascade (reviewed in Starr and Hilton, 1998). This was also shown for *Drosophila socs36E* (Callus and Mathey-Prevot, 2002; Karsten *et al.*, 2002). Expression of this gene is dependent on *upd* expression, the *Drosophila* JAK/STAT pathway ligand. *upd* and *socs36E* expression were detected in a complex, almost identical pattern during embryogenesis (Karsten *et al.*, 2002; Fig. 3.1).

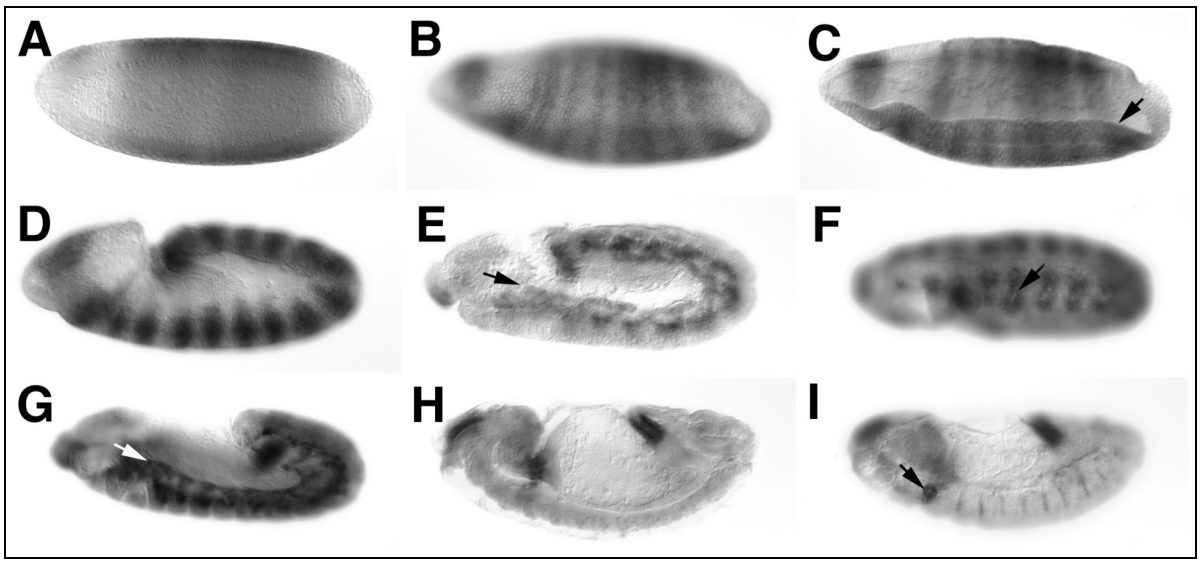

Fig. 3.1: Expression of *socs36E* during embryogenesis in *Drosophila* (modified after Karsten *et al.*, 2002). *socs36E* RNA *in situ* hybridization using a DIG labeled antisense probe with wild type embryos of all stages (see Material & Methods 2.8.1.2). Unless otherwise noted, embryos are shown anterior to the left and dorsal up. Embryonic staging was carried out according to Campos-Ortega and Hartenstein (1997). **(A)** Stage 5 embryo showed staining in the dorsal anterior head region and throughout most of the trunk. **(B)** & **(C)** The same stage 6 embryo focused laterally (B) and internally (C) showed *socs36E* expression in the head region, in two stripes flanking the presumptive cephalic furrow and in seven diffuse stripes

through the trunk region. Additionally, *socs36E* expression was detected in the invaginating mesoderm (arrow in C). **(D)** Early stage 9 embryo showed staining anterior to the ventral cephalic furrow, in the head region and in 14 stripes. **(E)** During stage 10 expression was transiently detected in three neuroblasts per-hemisegment. Additionally, *socs36E* began to be expressed in the leading edge cells (arrow in E). **(F)** Ventral view of a late stage 10 embryo showed a ring of expression surrounding the tracheal pits (arrow in F). **(G)** Stage 12 embryos showed expression in the trachea, the clypeolabrum and the hindgut (out of focus). *socs36E* expression in the leading edge cells (arrow in G) was strong and not detected using *upd* probes (not shown). **(H)** & **(I)** Expression in stage 14 embryos was detected in the clypeolabrum, proventriculus and hindgut. Expression in the anterior spiracle (arrow in I) and tracheal pits was maintained as stripes at more medial layers.

socs36E expression is first visible at stage 5 in a head stripe and a broad central domain (Fig. 3.1A). This pattern resolves first into seven and then 14 stripes (Fig. 3.1B - D). After transient expression in the presumptive mesoderm (Fig. 3.1C, arrow) and a subset of neuroblasts (Fig. 3.1E), *socs36E* expression is up-regulated in the developing tracheal pits (Fig. 3.1F, arrow) and the leading edge cells during dorsal closure (Fig. 3.1G, arrow). During stages 14/15 expression is limited to the inner clypeolabrum, the proventriculus, the hindgut and the anterior and medial spiracles (Fig. 3.1H & I).

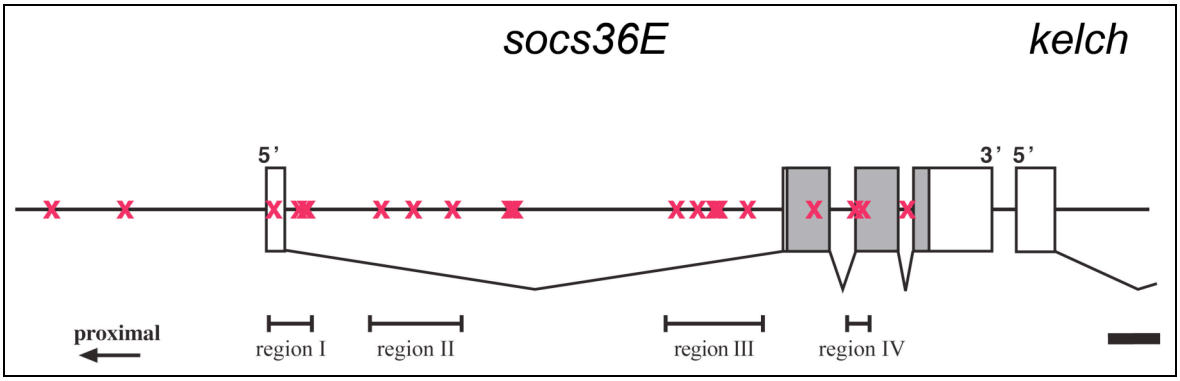

Fig. 3.2: **The genomic organization of the *socs36E* gene region (after Karsten *et al.*, 2002).** Intron/exon structure for *socs36E* as shown here is based on sequence analysis of the EST clones LD22121 and SD04320. The 3´ located gene *kelch* is shown as predicted by the Berkeley *Drosophila* genome project (FlyBase, 2003). Scale bar represents 1 kb. Coding sequences of *socs36E* are indicated in grey, untranslated sequences in white. Red crosses mark potential STAT92E DNA binding sites (as *in silico* determined). Regions I - IV cloned into a *lacZ*-reporter construct (*socs36E(region)-lacZ*) are marked by bars.

In the genomic region of *socs36E* covering 15.5 kb 22 potential STAT92E binding sites sharing the consensus sequence TTCNNNGAA (Yan *et al.*, 1996) were found (Karsten *et al.*, 2002; Fig. 3.2). To test, which of these potential sites STAT92E can bind and subsequently activate target gene expression *in vivo*, the genomic region was divided into several parts

containing three to five of the potential STAT92E binding sites (Fig. 3.2). These putative enhancer elements could then be used to mediate the expression of a reporter gene in order to monitor JAK/STAT pathway activity *in vivo* or in cultured cells. Therefore, the selected regions containing STAT92E recognition sites were cloned into a *Drosophila* transformation vector and fused to a basal promoter and the *lacZ* gene to produce a potential *socs36E-lacZ* reporter for JAK/STAT activity (see Material & Methods 2.3.1). Transgenic flies expressing *lacZ* under the control of the respective genomic sub-regions were generated. *lacZ* reporter gene expression in several transgenic lines was analyzed.

Anti-lacZ-antibody staining and RNA *in situ* hybridization using an antisense *lacZ* probe with embryos of all developmental stages were performed (see Material & Methods 2.8.1.3 & 2.8.1.2) to determine the transactivation activity of those genomic regions. It could be shown that different enhancer regions are active at distinct time points during embryogenesis. In addition, these different enhancer elements were also responsible for gene expression in different tissues. In none of the generated transgenic *w; P{w$^+$, socs36E-lacZ}* embryos the *lacZ* reporter gene was expressed in the complete wild type expression pattern of *socs36E* (compare Fig. 3.3 to Fig. 3.1). Unfortunately, due to technical difficulties it was not possible to assess the regulatory function of the entire region spanning 15.5 kb. Therefore, it cannot be ruled out that other regulatory elements inside and/or outside of this region contribute to the expression pattern of *socs36E*. In *w; P{w$^+$, socs36E(I)-lacZ}* embryos, region I containing five potential STAT92E binding sites was sufficient for *lacZ* expression in 14 stripes at stage 9 of development (Fig. 3.3A, panel 1). Expression was also visible in a subset of cells, presumably in the central nervous system (CNS) (Fig. 3.3, panel 2). Additionally, expression was detected during late embryogenesis in the tracheal pits and the fore- and hindgut primordium (Fig. 3.3, panel 3). A similar expression pattern could be detected in transgenic *w; P{w$^+$, socs36E(II)-lacZ}* embryos (Fig. 3.3B). During stage 9 *lacZ* expression could be observed in 14 stripes in the epidermis (Fig. 3.3B, panel 1), later also in the CNS (Fig. 3.3B, panel 2) and the hind- and foregut primordium (Fig. 3.3B, panel 3). The enhancer region III, comprising five putative STAT92E binding sites, was sufficient for *lacZ* expression in the leading edge cells during dorsal closure (Fig. 3.3C, panel 1 & 2) and also later during stages 13/14 in the fore- and hindgut in transgenic *w; P{w$^+$, socs36E(III)-lacZ}* embryos (Fig. 3.3C, panel 3). In *w; P{w$^+$, socs36E(IV)-lacZ}* embryos only a weak *lacZ* expression was detected, presumably in the CNS and the tracheal pits (Fig. 3.3D, panel 1 & 2). Taken together, regions I to IV contributed to the majority of expression domains of *socs36E* during embryogenesis.

However, the very early expression pattern of endogenous *socs36E* (see Fig. 3.1A - C) could not be detected in any of the transgenic *w; P{w$^+$, socs36E-lacZ}* embryos.

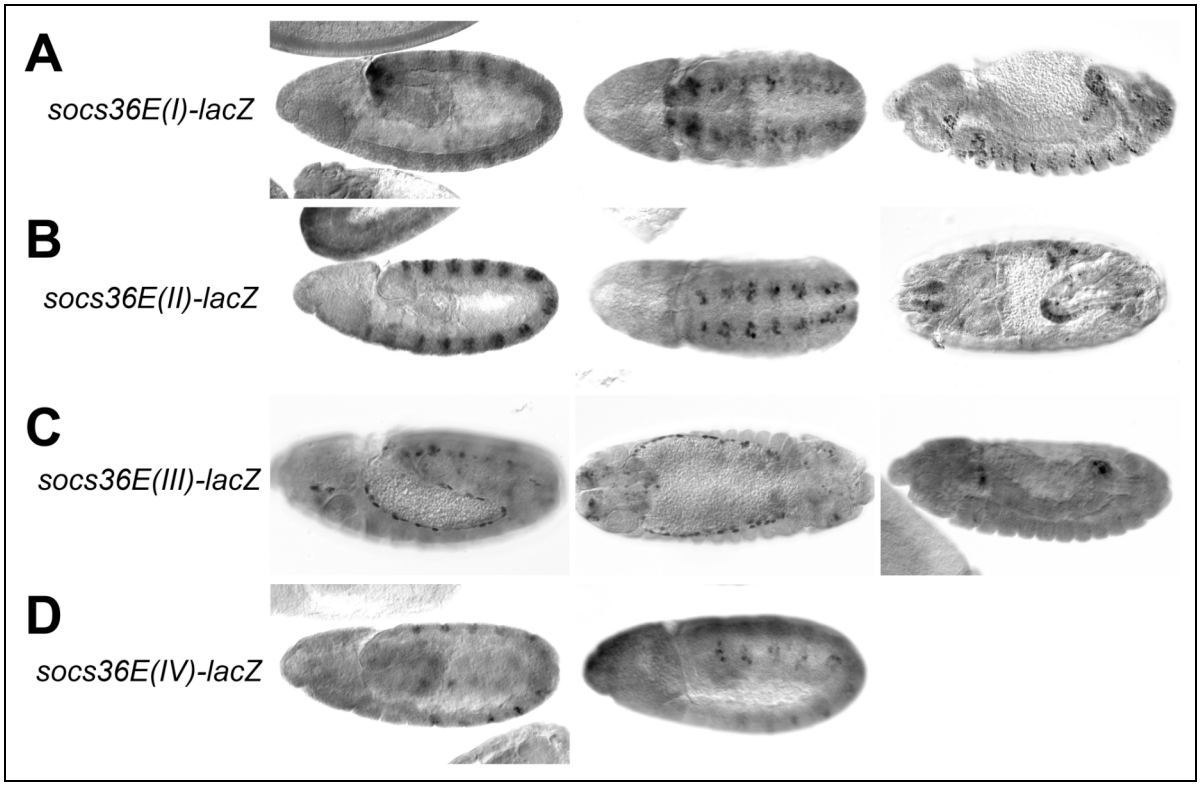

Fig. 3.3:**Transcriptional control of *socs36E*.** anti-LacZ antibody staining and *lacZ* RNA *in situ* hybridization using a DIG-labeled antisense probe with *socs36E-lacZ* transgene embryos of all stages (see Material & Methods 2.8.1.2). Embryos are shown anterior to the left in lateral or dorsal view. Embryonic staging was carried out according to Campos-Ortega and Hartenstein (1997). **(A)** In *w; P{w$^+$, socs36E(I)-lacZ}* embryos *lacZ* expression dependent on region I was detected in 14 stripes during stage 9 (panel 1) and the CNS (panel 2). At stage 14 *lacZ* expression was detected in tracheal pits and the fore- and hindgut (panel 3). **(B)** *lacZ* expression in *w; P{w$^+$, socs36E(II)-lacZ}* embryos dependent on region II was detected in 14 stripes during stage 9 (panel 1) and the CNS (panel 2). At stage 14 *lacZ* expression was detected in the fore- and hindgut (panel 3). **(C)** *lacZ* expression in *w; P{w$^+$, socs36E(III)-lacZ}* embryos dependent on region III was detectable in cells of the leading edge at stages 10 - 12 (panel 1 & 2) and the fore- and hindgut at stage 14 (panel 3). **(D)** *lacZ* expression in *w; P{w$^+$, socs36E(IV)-lacZ}* embryos dependent on region IV was detected in a subset of CNS cells (panel 1) and the tracheal pits (panel 2).

3.1.2 A luciferase reporter system to monitor JAK/STAT activity in cultured cells

The genomic region I was already shown to be sufficient for induction of *lacZ* reporter gene expression *in vivo* and to recapitulate major components of endogenous *socs36E* expression

(Fig. 3.3A). Furthermore, a bioinformatics survey concerning the number and spacing of the putative binding sites within the genomic region of *socs36E* resulted in a high probability that the sites of region I contribute to endogenous *socs36E* expression *in vivo* (Ho-Ryun Chung, personal communication). Therefore, region I containing five consensus STAT92E binding sites (Fig. 3.2) was the most promising candidate to generate a luciferase reporter construct to detect JAK/STAT pathway activity in cultured cells. For this purpose, region I was inserted into the pGL3-Promoter vector, which includes the firefly luciferase gene as inducible reporter for JAK/STAT activity (see Material & Methods 2.3.1). Alternatively, the 2x*Draf*STATwt-TATA-luc reporter (Kwon *et al.*, 2000) based on the *Drosophila raf* (*Draf*) gene promoter was tested. *Draf* is a transcriptional target of the JAK/STAT pathway and its promoter contains two consensus STAT92E recognition sites. The genomic region containing these binding sites was fused to the firefly luciferase gene (Kwon *et al.*, 2000). JAK/STAT activation by over-expression of the *Drosophila* JAK kinase Hopscotch (Hop) in cultured cells could be detected using this reporter construct (Kwon *et al.*, 2000). *Renilla* luciferase was constitutively expressed and used as normalization control. For this purpose, the coding region of *Renilla* luciferase was sub-cloned into the pAc5.1 vector to generate *pAct-Renilla* (Müller *et al.*, 2005; Karsten *et al.*, 2006). Three to four days after transfection, cells were lysed and luciferase activities were measured (see Material & Methods 2.4.3). Changes in the relative luciferase activity are a measure for JAK/STAT activity.

As shown in Fig. 3.4, the reporter system based on the *socs36E* enhancer region I did not respond to an activation of the JAK/STAT pathway in S2 cells (Schneider, 1972), neither by co-expressed Hop nor by the constitutively active allele $Hop^{Tumorous\text{-}lethal}$ (Hop^{Tuml}). Co-expression of a STAT92E-GFP fusion protein (see 3.1.3.1) together with the kinase did not result in the induction of firefly luciferase reporter gene expression (Fig. 3.4, compare column 1 to 2 & 3). By contrast, co-expression of STAT92E-GFP and Hop or Hop^{Tuml} resulted in pathway activation detectable by the 2x*Draf*STATwt-TATA-luc reporter (Kwon *et al.*, 2000). A three-fold increase in the relative reporter activity was observed after stimulation by both Hop and Hop^{Tuml} (Fig. 3.4, compare column 4 to 5 & 6). Using this reporter, relative luciferase activity could be increased to an eight-fold induction upon pathway stimulation in later experiments (see also Fig. 3.8). Therefore, in all following luciferase assays the reporter 2x*Draf*STATwt-TATA-luc (Kwon *et al.*, 2000) or the modified version *6x2xDrafLuc* (Müller *et al.*, 2005) were used to monitor JAK/STAT pathway activity in cultured *Drosophila* cells. The modified reporter version was constructed by multimerization of the genomic region comprising two STAT92E binding sites from the reporter 2x*Draf*STATwt-TATA-luc and

yielded approximately 50-fold induction of relative luciferase activity upon pathway stimulation (see Fig. 3.9).

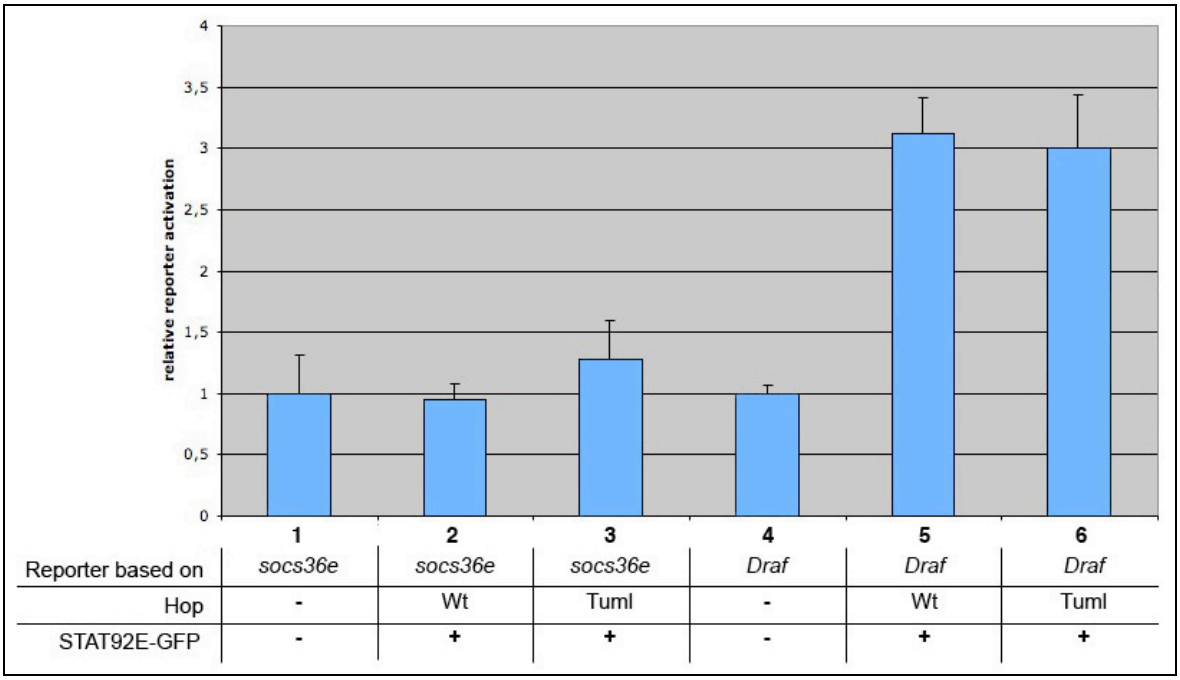

Fig. 3.4: **A cell culture reporter assay for JAK/STAT activity using the *socs36E* based reporter or the 2x*Draf*STATwt-TATA-luc reporter plasmid (Kwon *et al.*, 2000).** S2 cells (Schneider, 1972) co-expressing the indicated proteins (Wt: wild type), reporter plasmids and *Renilla* luciferase as normalization control. Lysates were used for dual luciferase reporter assays as described in Material & Methods 2.4.3.2. Relative activity (Firefly luciferase reporter activity / *Renilla* luciferase activity) is plotted. Mock-transfected cells were allocated the relative activity 1. Error bars represent standard deviations of four experiments. Induction of relative reporter activity was not detectable using the reporter construct based on the *socs36E* enhancer region I (columns 1 - 3), whereas co-expression of STAT92E-GFP and Hop or Hop^{Tuml} resulted in a three-fold increase of relative reporter activity (colums 4 - 6) using the 2x*Draf*STATwt-TATA-luc reporter construct (Kwon *et al.*, 2000).

3.1.3 *Drosophila* STAT: STAT92E

STAT92E plays a central role in the *Drosophila* JAK/STAT pathway. This protein is responsible for the signal transduction from the membrane-associated ligand/receptor/kinase-complex through the cytoplasm into the nucleus. For this purpose, it has to leave the cytoplasm and enter the nucleus to subsequently accomplish its function in activating target gene expression.

3.1.3.1 Sub-cellular localization of STAT92E-GFP

In order to follow the sub-cellular localization of STAT92E, the fusion protein STAT92E-GFP was generated (see Material & Methods 2.3.1). Following activation STAT dimers accumulate in the nucleus of mammalian cells (Brierley and Fish, 2005). Therefore, an accumulation of STAT92E-GFP in the nucleus of transfected cultured *Drosophila* cells should be detectable upon pathway stimulation with the constitutively active kinase Hop^{Tuml}.

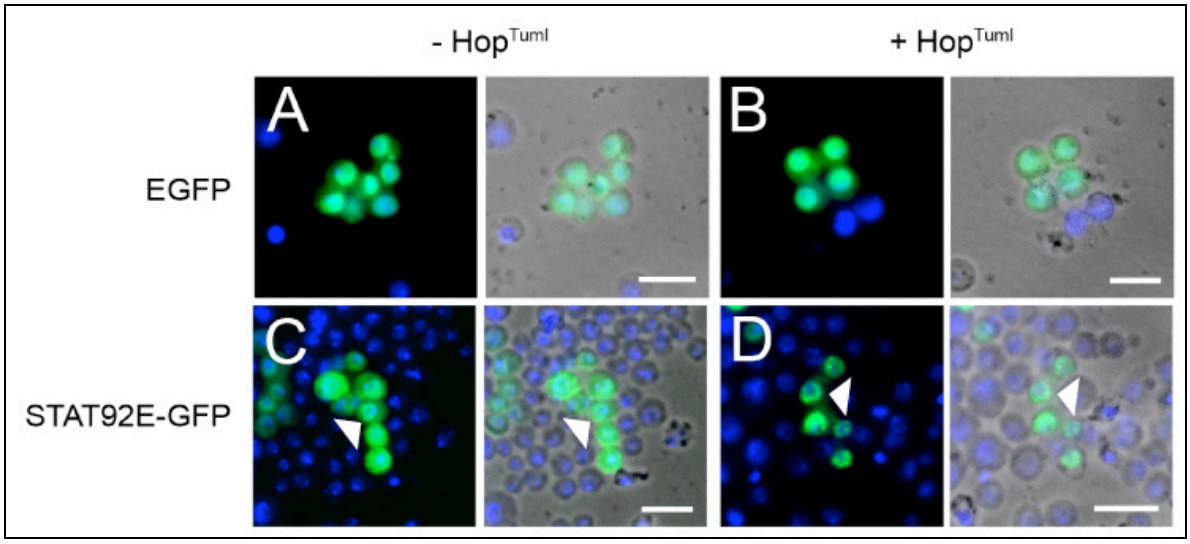

Fig. 3.5: **Sub-cellular localization of STAT92E-GFP.** Kc_{167} cells (Cherbas *et al.*, 1977) expressing the indicated proteins. Columns 1 & 3 show GFP fluorescence and DAPI staining of DNA reflecting the localization of GFP fusion proteins (green) and the nuclei (blue), respectively. Columns 2 & 4 show columns 1 & 3 overlaid on a brightfield image of the cells. Scale bars represent 20 µM. **(A)** EGFP is distributed throughout the cells and **(B)** does not respond to stimulation by Hop^{Tuml}. **(C)** STAT92E-GFP protein is localized in the cytoplasm and the nucleus of non-stimulated Kc_{167} cells (arrows). **(D)** Pathway stimulation by co-expression with Hop^{Tuml} resulted in an accumulation of STAT92E-GFP in the nucleus and depletion from the cytoplasm (arrows).

STAT92E-GFP was ectopically expressed via the Gal4/UAS system (Brand and Perrimon, 1993) in *Drosophila* Kc_{167} cells (Cherbas *et al.*, 1977). These cells were analyzed for the sub-cellular localization of the GFP signal using a fluorescence microscope. As a control, EGFP alone was expressed. It was distributed throughout the cell (Fig. 3.5A) including nuclear localization, which was probably due to unrestricted transport because of the little molecular weight of EGFP (30 kDa). Stimulation of the JAK/STAT pathway by co-expression with Hop^{Tuml} did not alter the localization of EGFP (Fig. 3.5B) showing that this protein did not respond to JAK/STAT signaling. In non-stimulated cells STAT92E-GFP was distributed throughout the cell (arrows in Fig. 3.5C). Additionally, STAT92E-GFP was not excluded

from the nucleus. This suggests a low basal level of STAT92E activation or activation-independent transport into the nucleus similar to other STAT proteins (reviewed in Meyer and Vinkemeier, 2004). STAT92E-GFP, activated by co-expressed Hop^{Tuml}, accumulated in the nucleus, mainly visible by the clearance of the cytoplasm (arrows in Fig. 3.5D). The detected fluorescence reflected the putative activation state of STAT92E-GFP, a finding consistent with activation-dependent nuclear accumulation of vertebrate STATs (Brierley and Fish, 2005).

3.1.3.2 Activation-dependent phosphorylation of STAT92E-GFP

Phosphorylation of a conserved C-terminal tyrosine residue is essential for the function of STAT proteins (Shuai *et al.*, 1993a). Therefore, the phosphorylation state of the conserved tyrosine residue Y704 of *Drosophila* STAT92E was tested using the STAT92E phospho-specific antibody pSTAT92E (Li *et al.*, 2003).

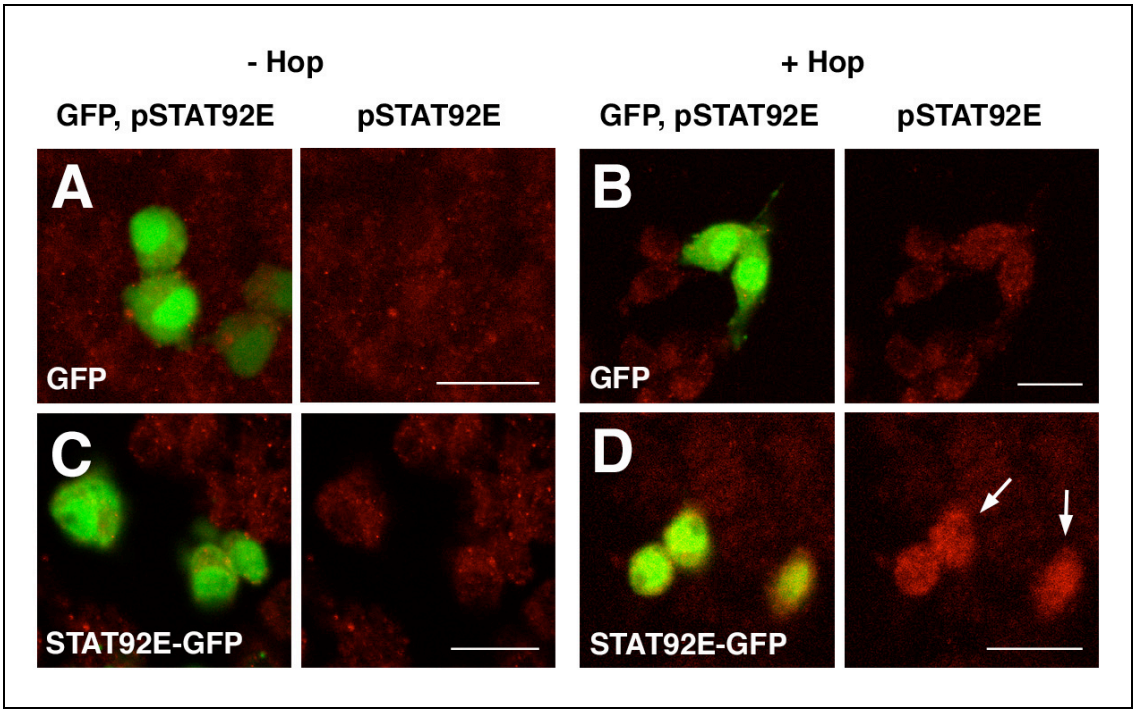

Fig. 3.6: **Phosphorylation state of STAT92E-GFP.** S2 cells (Schneider, 1972) expressing the indicated proteins, visualized by GFP fluorescence (green) and anti-pSTAT92E indirect immunofluorescence (red). Columns 1 & 3 show the overlay of the EGFP and anti-pSTAT92E signal. Columns 2 & 4 show the anti-pSTAT92E signal alone. Scale bars represent 16 µM. **(A)** In cells expressing EGFP pSTAT92E staining was not detectable under non-stimulated conditions. **(B)** Co-expression of EGFP with Hop did not alter levels of pSTAT92E staining. **(C)** In cells expressing STAT92E-GFP alone pSTAT92E staining was

not detectable under non-stimulated conditions. **(D)** STAT92E-GFP and Hop co-expression resulted in increased levels of pSTAT92E (arrows).

In *Drosophila* S2 cells (Schneider, 1972) expressing EGFP the anti-pSTAT92E antibody showed a low level of background staining (Fig. 3.6A). In cells co-expressing EGFP and Hop no increase in anti-pSTAT92E staining was detectable compared to the non-stimulated state (Fig. 3.6, compare A to B). This suggested that endogenous STAT92E was not present or detectable in these cells by this assay. STAT92E-GFP expression alone did not result in anti-pSTAT92E staining above background level (Fig. 3.6, compare A to C) as expected for an antibody displaying STAT92E phospho-Y704-specific binding activity. However, when STAT92E-GFP was activated by co-expressed Hop, an increased level of pSTAT92E could be detected (arrows in Fig. 3.6D). Thus, activation of JAK/STAT signaling by the kinase Hop caused Y704 phosphorylation of STAT92E-GFP in cultured *Drosophila* cells.

3.1.3.3 DNA binding ability of STAT92E-GFP

It is known that STAT proteins bind to specific DNA sequences in promoters of target genes (Stark *et al.*, 1998). The DNA binding ability of the fusion protein STAT92E-GFP to its consensus DNA recognition site TTCCCGGAA (Yan *et al.*, 1996) was tested in an electro-mobility shift assay (EMSA, see Material & Methods 2.5.3). In this approach the presence of STAT92E-GFP dimers, competent to bind DNA *in vitro*, can be detected with high sensitivity. Therefore, whole lysates of S2 cells (Schneider, 1972) ectopically expressing STAT92E-GFP alone or together with Hop were tested for their ability to bind double-stranded radio-labeled oligonucleotides containing the STAT92E consensus DNA recognition site TTCCCGGAA. Lysates of non-transfected cells did not show any binding activity (Fig. 3.7A, lane 1). Thus, the S2 cells used in this experiment did not provide endogenous levels of JAK/STAT activity detectable by this assay. This was also supported by the fact that in lysates of cells expressing STAT92E-GFP alone no mobility shift was detectable (Fig. 3.7B, lane 1). Hence, the upstream components of the pathway were either not present or not endogenously activated to a detectable extent in these cells, as already shown in 3.1.3.2. However, in lysates of S2 cells co-expressing STAT92E-GFP together with Hop DNA binding activity was detectable as manifested by a shifted band in this EMSA experiment (Fig. 3.7A, lane 2). This band could be super-shifted by addition of an anti-GFP antibody (arrow in Fig. 3.7A, lane 3) showing the existence of GFP in this complex. This demonstrated

that the shifted band represented STAT92E-GFP bound to the radio-labeled oligonucleotide containing the STAT92E recognition site. The specificity of the binding process could be confirmed in a competition assay. Addition of increasing amounts of excess unlabeled oligonucleotides containing the consensus binding site resulted in an attenuation of the shifted band (Fig. 3.7B, compare lane 2 to lanes 3 - 5). In contrast, oligonucleotides containing a mutated binding site (TTGCCGCAA) were not able to compete with the radio-labeled wild type binding site. Even when double-stranded oligonucleotides in 100x excess were added, the signal of the shifted band was not affected (Fig. 3.7B, compare lane 2 to lanes 6 - 8). This indicated that STAT92E-GFP specifically bound the STAT92E DNA consensus recognition site.

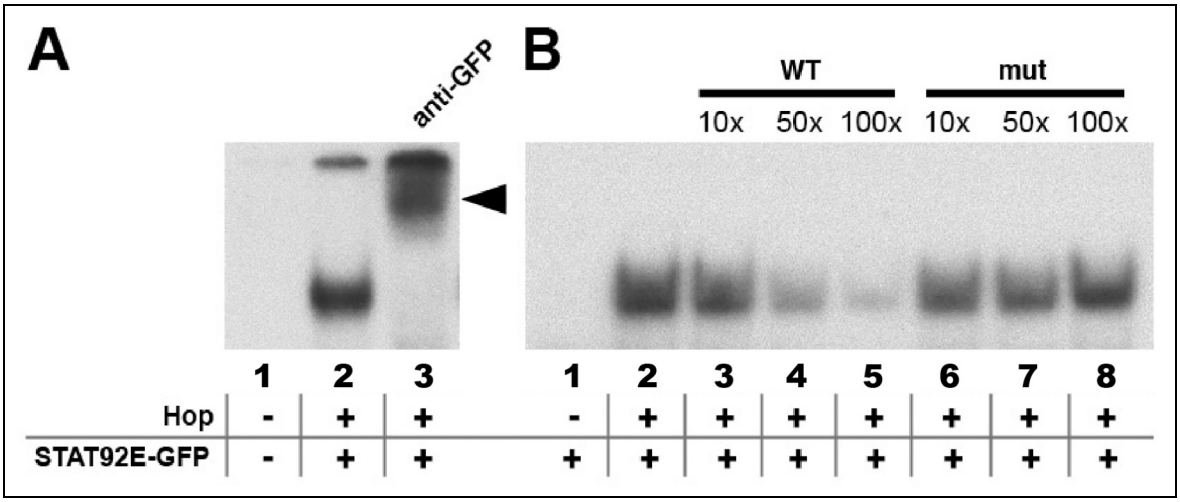

Fig. 3.7: **DNA binding ability of STAT92E-GFP.** Indicated proteins were co-expressed in S2 cells (Schneider, 1972). Crude cell lysates were tested for DNA binding ability to radio-labeled oligonucleotides containing the STAT92E consensus DNA recognition site TTCCCGGAA (see Material & Methods 2.5.3). **(A)** STAT92E-GFP bound to the STAT92E consensus DNA recognition site TTCCCGGAA when co-expressed with Hop (lane 2). This shifted band could be super-shifted by an anti-GFP antibody (arrow head in lane 3). Extra bands at the top of lanes 2 & 3 were loading artifacts. **(B)** Unlabeled double-stranded oligonucleotides containing the STAT92E consensus recognition site TTCCCGGAA (WT) could compete with radio-labeled double-stranded oligonucleotides for binding (lanes 3 - 5) while double-stranded oligonucleotides containing a mutated consensus recognition sequence TTGCCGCAA (mut) did not compete (lanes 6 - 8). Oligonucleotides were added in 10x, 50x, 100x excess.

Taken together, I could demonstrate that STAT92E-GFP complexes, which show binding specificity for the previously determined DNA recognition site (Yan *et al.*, 1996), can be detected in EMSA experiments. Furthermore, I could show that STAT92E-GFP mediated DNA binding to this site occurred only after activation by Hop in these cells. STAT92E-GFP

did not show any specific DNA binding activity to its DNA recognition site under non-stimulated conditions.

3.1.3.4 Transcriptional activation by STAT92E-GFP

Although tyrosine phosphorylation, nuclear translocation and DNA binding are required for STAT activity, the key role for a transcription factor is its ability to elicit the expression of pathway target genes. To test the functionality of STAT92E-GFP and its ability to induce target gene expression compared to wild type STAT92E, the luciferase reporter assay for JAK/STAT activity described above (see 3.1.2) was used.

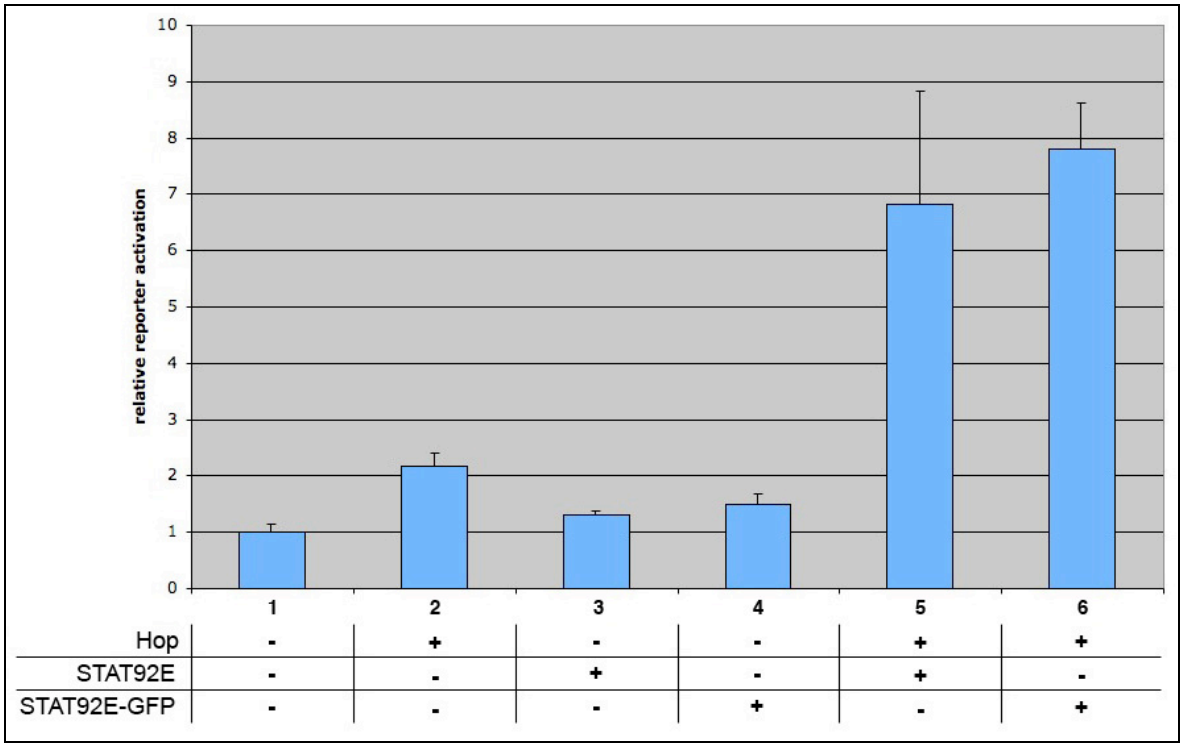

Fig. 3.8: JAK/STAT pathway activity transduced by STAT92E or the fusion protein STAT92E-GFP. S2 cells (Schneider, 1972) co-expressing the indicated proteins, the 2x*Draf*STATwt-TATA-luc reporter (Kwon *et al.*, 2000) and *Renilla* luciferase as normalization control. Lysates were used for dual luciferase reporter assay as described in Material & Methods 2.4.3.2. Relative activity (Firefly luciferase reporter activity / *Renilla* luciferase activity) is plotted. Mock-transfected cells were allocated the relative activity 1. Error bars represent standard deviations of three experiments. Expression of each Hop, STAT92E or STAT92E-GFP alone did not result in (columns 3 & 4) or only in mild induction of relative reporter activity (column 2). Co-expression of Hop and STAT92E or STAT92E-GFP resulted in a seven- to eight-fold increase of relative reporter activity (columns 5 & 6).

Using the 2x*Draf*STATwt-TATA-luc reporter (Kwon *et al.*, 2000) in S2 cells (Schneider, 1972), it was shown that expression of Hop resulted in a slight induction of luciferase reporter activity (Fig. 3.8, column 2) suggesting low levels of endogenous STAT92E present in these cells which was activated by ectopic expressed Hop. No firefly luciferase activity above background levels was detectable after expression of STAT92E or STAT92E-GFP alone (Fig. 3.8, columns 3 & 4). This indicated that no detectable endogenous pathway activity was present in these cells. However, co-expression of STAT92E or STAT92E-GFP together with Hop resulted in a seven- to eight-fold response (Fig. 3.8, columns 5 & 6). This clearly showed that the C-terminal GFP-tag did not alter the function of STAT92E in this assay system. Both, STAT92E and the fusion protein STAT92E-GFP were able to activate reporter gene expression (Fig. 3.8, compare columns 5 & 6) with no significant difference.

3.1.3.5 RNAi as a tool to identify potential JAK/STAT pathway interactors

In further experiments a modified form of the reporter, *6x2xDrafLuc*, was also used to detect JAK/STAT activity. In this vector the STAT92E binding sites were multimerized to result in a reporter with 12 binding sites (Müller *et al.*, 2005). Activation of the JAK/STAT pathway by expression of an Upd-GFP fusion protein (Müller *et al.*, 2005) resulted in an approximately 50-fold induction of luciferase reporter activity (Fig. 3.9, column 6).

It was shown that addition of dsRNA is a potent tool to sequence-specifically silence gene expression (Fire *et al.*, 1998). During this procedure dsRNA is internalized by the cells and recognized by the Dicer enzyme. This enzyme cleaves dsRNA into precisely sized fragments of ~22 nucleotides, so called small interfering RNAs (siRNAs). These siRNAs join the RNA-induced silencing complex (RISC) and guide this complex to homologous mRNA substrates, which then are specifaclly degraded (reviewed in Hannon, 2002).

This method of gene silencing was used to knock down endogenous mRNA of pathway interacting genes in cultured cells (see Material & Methods 2.4.4). For this purpose, cells expressing Upd-GFP were incubated with dsRNA added to the culture medium covering about 500bp of the ORF of the respective target genes. Treatment with *Dome* and *stat92E* dsRNA decreases the relative luciferase reporter activity back to less than half the stimulated level (Fig. 3.9, compare column 6 to 8 & 9). This showed the important role of these proteins for Upd signal transduction to the nucleus. Furthermore, another component of the pathway, the negative regulator SOCS36E, was tested for its effect on JAK/STAT signaling in this luciferase assay. When *socs36E* mRNA was knocked down by addition of dsRNA, a clear

increase of reporter activity was detected in both non-stimulated and Upd stimulated cells (Fig. 3.9, columns 5 & 10). This suggested a low level activity of the JAK/STAT pathway in these cells, which is normally counteracted by SOCS36E. Addition of *lacZ* dsRNA as a negative control did not alter the luciferase activity (Fig. 3.9, columns 2 & 7).

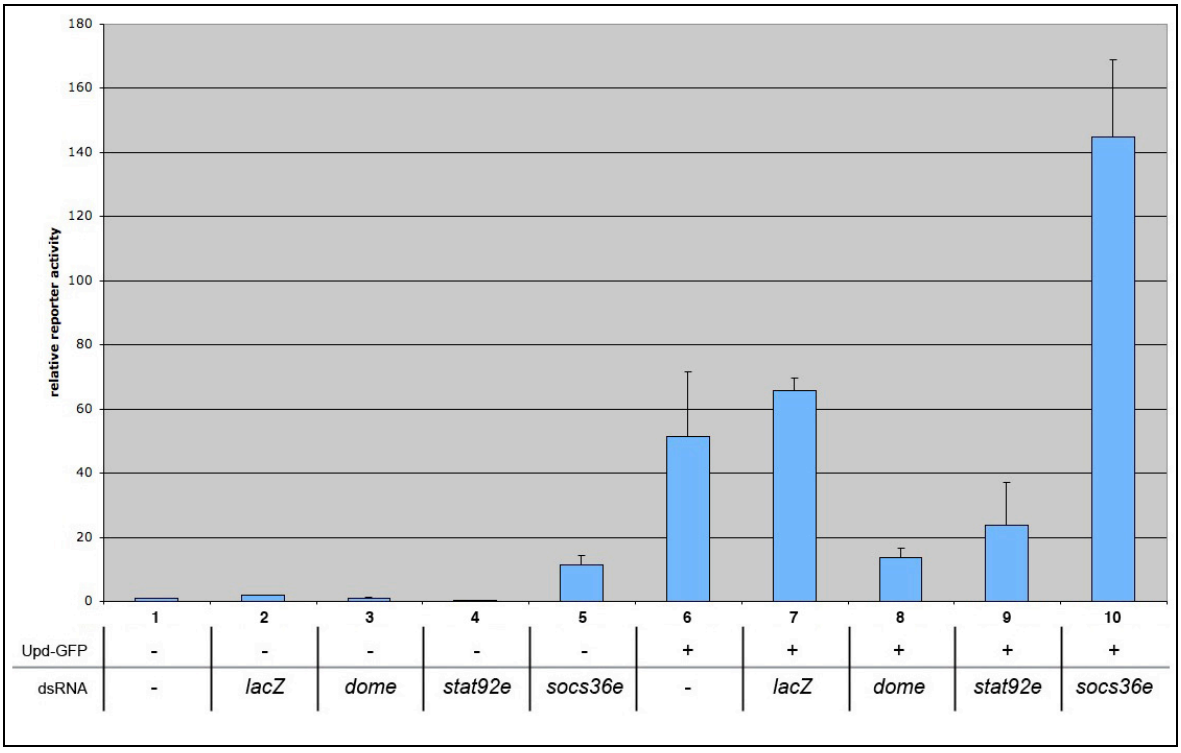

Fig. 3.9: Transcriptional activation of the JAK/STAT pathway via Upd-GFP. Kc_{167} cells (Cherbas *et al.*, 1977) co-expressing the indicated proteins, the *6x2xDrafLuc* reporter (Müller *et al.*, 2005) and *Renilla* luciferase as normalization control. Cells were incubated with the indicated dsRNA (1.5 µg/10^5 cells) until lysis. Lysates were used for dual luciferase reporter assays as described in Material & Methods 2.4.3.2. Relative activity (Firefly luciferase reporter activity / *Renilla* luciferase activity) is plotted. Mock-transfected cells were allocated the relative activity 1. Error bars represent standard deviations of four experiments. Expression of Upd-GFP (Müller *et al.*, 2005) resulted in a 50-fold increase of relative reporter activity (column 6). Knocking down *dome* or *stat92E* mRNA repressed relative reporter activity (columns 8 & 9) showing the importance of these genes for JAK/STAT signaling. SOCS36E acted as repressor of JAK/STAT signaling. Knocking down *socs36E* mRNA resulted in a strong increase of relative reporter activity (columns 5 & 10).

In summary, these results showed that changes in JAK/STAT signaling activity were responsible for the alteration of the firefly luciferase reporter activity. STAT92E-GFP was fully functional and the 2x*Draf*STATwt-TATA-luc reporter (Kwon *et al.*, 2000) and its modified version *6x2xDrafLuc* (Müller *et al.*, 2005) can be used to detect JAK/STAT activity in cultured *Drosophila* cells (Fig. 3.8, Fig. 3.9 and see also Fig. 3.4). Additionally, it was

shown that RNAi could be used to alter luciferase reporter activity by silencing expression of JAK/STAT pathway components or regulators. This provides a tool to test for the interaction of candidate genes with this signaling cascade. Knocking down negative regulators resulted in an increase, whereas knocking down positive regulators led to a decrease of luciferase reporter activity as it was shown for *socs36E*, *dome* and *stat92E*, respectively (Fig. 3.9).

3.2 A constitutive active allele of STAT

Ariyoshi and colleagues described a gain-of-function allele of mouse STAT5A (Ariyoshi *et al.*, 2000), which contains an asparagine to histidine substitution (N642H) in its SH2 domain. This mutant STAT5A^{N642H}-Flag fusion protein was identified by conferring autonomous cell growth on interleukin-3-dependent Ba/F3 cells. The gain-of-function characteristic was presumably due to constitutive phosphorylation of the conserved residue Y694 and thereby activation of STAT5A^{N642H}-Flag (Ariyoshi *et al.*, 2000).

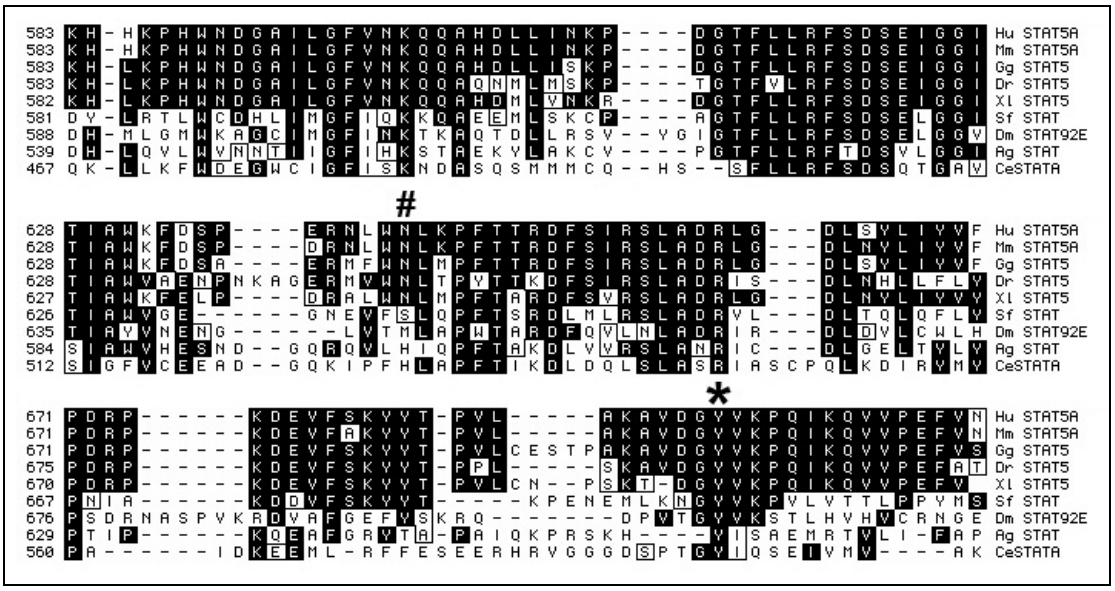

Fig. 3.10: **Alignment of the C-terminal region containing the SH2 domain of several STAT proteins.** Identical amino acids are shaded black. Protein sequence identities are listed in Materials & Methods 2.6. Residues aligning to N642 of mouse STAT5A (M647 of *Drosophila* STAT92E) and to the conserved tyrosine residue (Y704 of *Drosophila* STAT92E) are marked by the pound sign (#) and the asterisk (*), respectively. Hu = *Homo sapiens*, Mm = *Mus musculus*, Gg = *Gallus gallus*, Dr = *Danio rerio*, Xl = *Xenopus laevis*, Sf = *Spodoptera frugiperda*, Dm = *Drosophila melanogaster*, Ag = *Anopheles gambiae*, Ce = *Caenorhabditis elegans*.

Analysis of mutant pathway components can help to give an insight into the mode of signal transduction and also to characterize pathway co-regulators. Activation of signaling by constitutive active alleles of STAT, independent of classical pathway stimulation via ligand binding, can be used to classify pathway regulators in up- or downstream effectors of STAT by the analysis of epistatic effects.

In order to generate a constitutively active allele of *Drosophila* STAT92E, the residue corresponding to N642 of mouse STAT5A was identified. For this purpose, protein alignments of several STAT proteins were performed using the clustal alignment algorithm of MegAlign software (DNASTAR Inc.; Higgins and Sharp, 1989). These alignments showed that methionine at position 647 (M647) of STAT92E corresponds to N642 of STAT5A (Fig. 3.10). Interestingly, STATs of lower organisms like the nematode *Caenorhabditis elegans* and the mosquito *Anopheles gambiae* already have a histidine at this position. So far no JAK-like proteins have been identified in those organisms. This suggests an alternative mode of pathway activation independent of phosphorylation by JAK proteins.

3.2.1 Generation of mutant STAT92E-GFP variants

Having shown that the STAT92E-GFP fusion protein is fully functional in cell culture assays, such fusion proteins were used to analyze various STAT92E mutants. Residue M647 of STAT92E-GFP was mutated to a histidine (M647H) in order to generate a constitutively active allele of *Drosophila* STAT92E corresponding to STAT5A^{N642H}-Flag (Ariyoshi *et al.*, 2000). Furthermore, the conserved tyrosine residue Y704, target of JAK-mediated phosphorylation, was mutated to phenylalanine (Y704F) to generate a non-functional STAT92E-GFP. In addition, the double mutant STAT92E^{M647H,Y704F}-GFP was generated (see Material & Methods 2.3.3). These STAT92E-GFP mutant proteins were analyzed for their function using the assays described above.

3.2.2 Sub-cellular localization of STAT92E-GFP variants

STAT92E-GFP accumulated in the nucleus after stimulation with Hop (see Fig. 3.5). The mutant STAT92E^{Y704F}-GFP protein was localized throughout the cell similar to non-stimulated STAT92E-GFP (arrows in Fig. 3.11C, compare to Fig. 3.5C). However, after stimulation by Hop^{Tuml}, STAT92E^{Y704F}-GFP was not able to accumulate in the nucleus, but remained localized throughout the cell (arrows in Fig. 3.11D) in contrast to STAT92E-GFP

(see Fig. 3.5D). This result emphasized the importance of the conserved tyrosine residue Y704, mutation of which abolished activation-dependent nuclear accumulation.

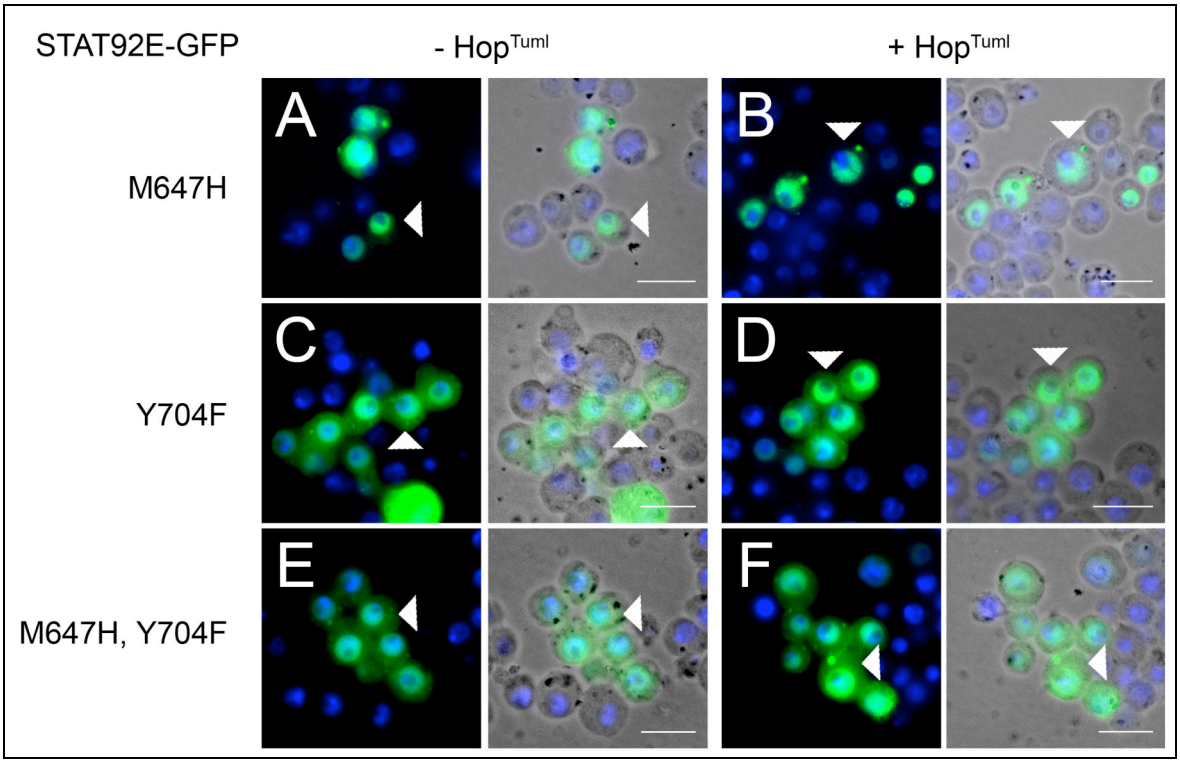

Fig. 3.11: **Sub-cellular localization of STAT92E-GFP variants.** Kc_{167} cells (Cherbas *et al.*, 1977) co-expressing the indicated proteins. Columns 1 & 3 show GFP fluorescence and DAPI staining of DNA reflecting the localization of GFP fusion proteins (green) and the nuclei (blue), respectively. Columns 2 & 4 show columns 1 & 3 overlaid on a brightfield image of the cells. Scale bars represent 20 µM. **(A)** $STAT92E^{M647H}$-GFP accumulated in the nuclei of non-stimulated cells (arrows). **(B)** In cells co-expressing Hop^{Tuml} $STAT92E^{M647H}$-GFP was also located in the nuclei of the cells (arrows). **(C)** & **(D)** $STAT92E^{Y704F}$-GFP did not accumulate in the nuclei of the cells under both non-stimulated and stimulated conditions (arrows). Y704F mutation abolished nuclear translocation of STAT92E-GFP. **(E)** & **(F)** Double mutant $STAT92E^{M647H,Y704F}$-GFP did also not accumulate in the nuclei of the cells under both non-stimulated and stimulated conditions (arrows). Y704F mutation was sufficient to abolish nuclear translocation of $STAT92E^{M647H}$-GFP.

Strikingly, expression of $STAT92E^{M647H}$-GFP resulted in fluorescence that was nuclear enriched even in the absence of co-expressed Hop^{Tuml} when compared to non-stimulated STAT92E-GFP (arrows in Fig. 3.11A, compare to Fig. 3.5C). This effect was even stronger under stimulated conditions, when almost no fluorescence was detectable in the cytoplasm anymore (arrows in Fig. 3.11B). Thus, the mutation M647H of STAT92E-GFP enabled nuclear enrichment of this protein even without activation by co-expressed Hop^{Tuml}. This result was a first indication that $STAT92E^{M647H}$-GFP is a constitutively active allele of

STAT92E as it was shown for the corresponding mouse STAT5A^{N642H}-Flag (Ariyoshi *et al.*, 2000). In the double mutant STAT92E^{M647H,Y704F}-GFP, Y704F mutation was sufficient to prevent the nuclear enrichment of the protein, as it was shown for the single mutant STAT92E^{Y704F}-GFP (arrows in Fig. 3.11E & F). This demonstrated the essential role of this tyrosine residue for the sub-cellular localization of STAT92E^{M647H}-GFP.

3.2.3 Phosphorylation state of STAT92E-GFP variants

After stimulation by Hop, phosphorylated STAT92E-GFP could be detected with anti-pSTAT92E antibodies (see Fig. 3.6; Li *et al.*, 2003).

Strikingly, STAT92E^{M647H}-GFP was inherently phosphorylated on Y704 even under non-stimulated conditions as indicated by elevated levels of anti-pSTAT92E staining (arrows in Fig. 3.12A, compare to Fig. 3.6C). Co-expression with Hop resulted in an even stronger signal (Fig. 3.12, compare A to B). These results suggested that STAT92E^{M647H}-GFP was, at least partly, constitutively phosphorylated on Y704. Additionally, this phosphorylation was independent of activation by Hop. However, stimulation by Hop could also contribute to Y704 phosphorylation of STAT92E^{M647H}-GFP as shown by the increased level of pSTAT92E under stimulated conditions (Fig. 3.12, compare A to B). These results were consistent with the gain-of-function characteristics of mouse STAT5A^{N642H}-Flag (Ariyoshi *et al.*, 2000). STAT92E-GFP mutants containing the Y704F substitution were not phosphorylated to an extent detectable by anti-pSTAT92E antibody (Fig. 3.12C – F). An increased level of pSTAT92E staining could not be observed after pathway stimulation by Hop, neither for the STAT92E^{Y704F}-GFP single nor for the STAT92E^{M647H,Y704F}-GFP double mutant (Fig. 3.12D & F). This confirmed that Y704 is a target of Hop. Together with the results showing defects in the activation-dependent nuclear accumulation for STAT92E-GFP Y704 mutants (see Fig. 3.11C - F), this demonstrated that Y704 phosphorylation is essential for proper sub-cellular localization of STAT92E-GFP.

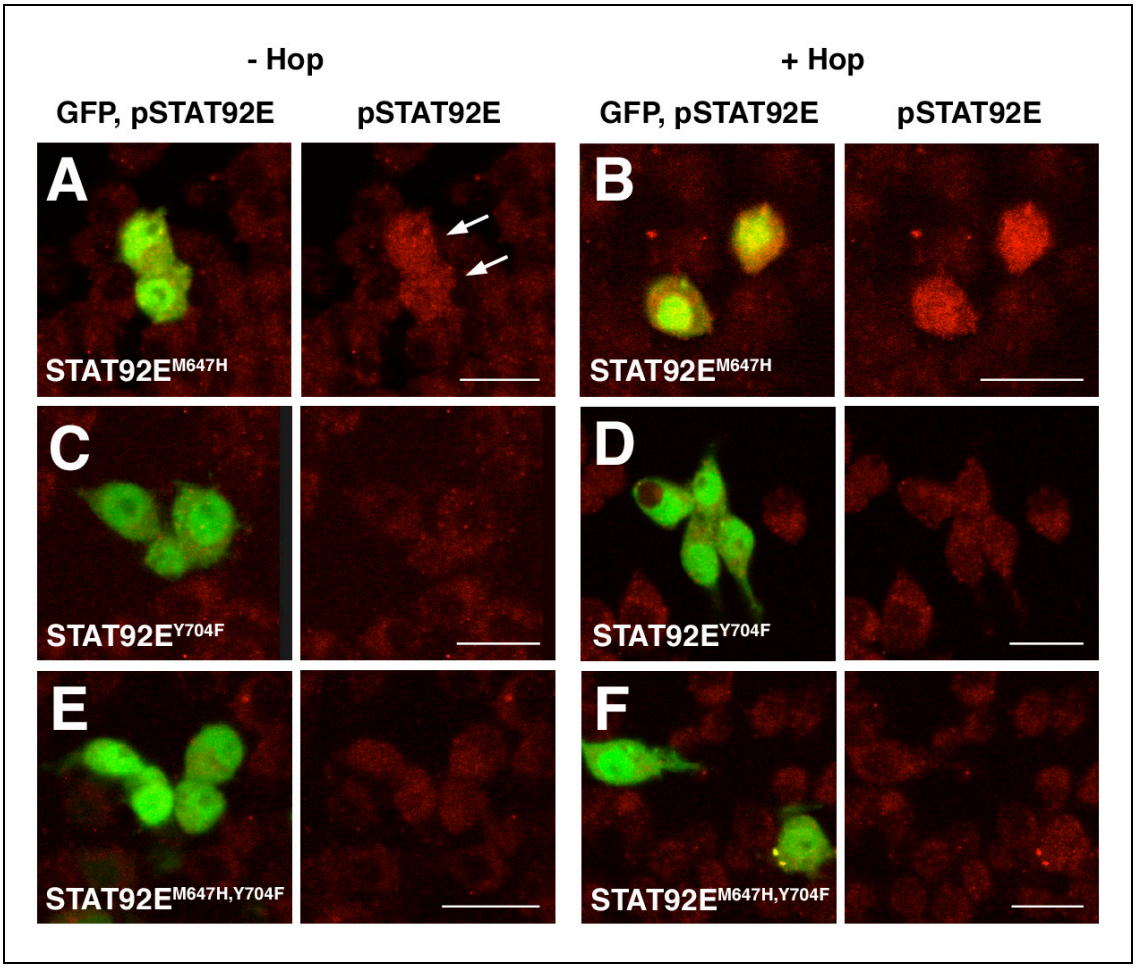

Fig. 3.12: **Phosphorylation state of STAT92E-GFP variants.** S2 cells (Schneider, 1972) expressing the indicated proteins. STAT92E-GFP fusion proteins were visualized by GFP fluorescence (green) and anti-pSTAT92E indirect immunofluorescence (red). Columns 1 & 3 show the overlay of EGFP and anti-pSTAT92E signal. Columns 2 & 4 show anti-pSTAT92E signal alone. Scale bars represent 16 µM. **(A)** In cells expressing STAT92E^{M647H}-GFP raised levels of pSTAT92E staining were detectable under non-stimulated conditions (arrows). **(B)** Co-expression of STAT92E^{M647H}-GFP and Hop resulted in increased levels of pSTAT92E compared to (A). **(C)** - **(F)** Elevated levels of pSTAT92E could not be detected in cells expressing either STAT92E^{Y704F}-GFP single or STAT92E^{M647H,Y704F}-GFP double mutant both under non-stimulated and stimulated conditions.

3.2.4 DNA binding ability of STAT92E-GFP variants

In EMSA experiments STAT92E-GFP could specifically bind radio-labeled oligonucleotides containing the STAT92E consensus DNA recognition site TTCCCGGAA after stimulation by Hop (see Fig. 3.7).

Before testing the DNA binding ability of the STAT92E-GFP mutants, the relative protein levels were examined by Western blotting of whole cell lysates (see Material & Methods 2.5.2). The results showed that all STAT92E-GFP variants were expressed in S2 cells

(Schneider, 1972) in comparable levels and were stable with no major degradation products detected (Fig. 3.13A, lower panel). Using NIH Image software the luminographs of these Western blots were quantified, and protein levels were normalized before EMSA analysis was performed.

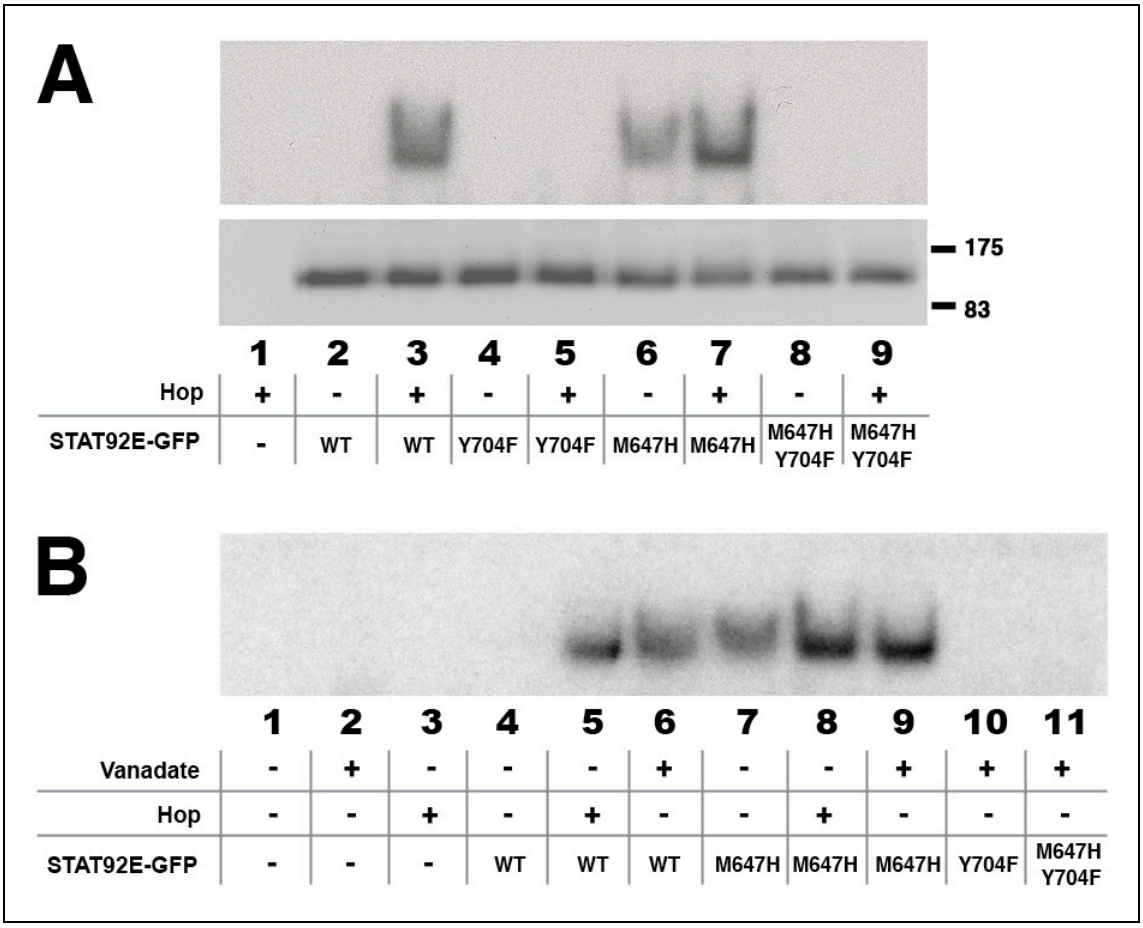

Fig. 3.13: **DNA binding ability of STAT92E-GFP variants.** S2 cells (Schneider, 1972) expressing the indicated proteins. Crude cell lysates were tested for DNA binding ability to radio-labeled oligonucleotides containing the STAT92E consensus DNA recognition site TTCCCGGAA (see Material & Methods 2.5.3). **(A)** STAT92E-GFP mutants, co-expressed without or with Hop, bound to radio-labeled oligonucleotides containing the STAT92E consensus DNA recognition site TTCCCGGAA. Expression of Hop or STAT92E-GFP alone was not sufficient for detecting a band shift (lane 1 or 2) whereas co-expression of both proteins together resulted in a shifted band (lane 3). Mutation of Y704 in the single or the double mutant abolished DNA binding completely (lanes 4 & 5, 8 & 9). STAT92E^{M647H}-GFP mutant proteins could bind to DNA even in the absence of Hop stimulation (lane 6). However, DNA binding was stronger when Hop was co-expressed (lane 7). Extracts used for EMSA were also analyzed by Western blotting using anti-GFP antibody. Proteins were stable and equally loaded (lower panel). **(B)** STAT92E-GFP mutants co-expressed without or with Hop or treated with 0.1 mM sodium-ortho-vanadate for 45 minutes before cell lysis. Binding to radio-labeled oligonucleotides containing the STAT92E consensus DNA recognition site TTCCCGGAA was visualized by the shifted band. STAT92E-GFP could be stimulated to bind DNA in the absence of Hop following treatment with sodium-ortho-vanadate (lane 6)

and gave a signal similar to that produced by the STAT92E^{M647H}-GFP mutant under non-stimulated conditions (lane 7). Y704F mutant proteins could not be stimulated by sodium-ortho-vanadate (lanes 10 & 11).

Expression of neither Hop (Fig. 3.13A, lane 1) nor STAT92E-GFP (Fig. 3.13A, lane 2) alone resulted in a detectable shifted band. Thus, the S2 cells used in this experiment did not provide endogenous levels of Hop activity and did not express levels of STAT92E detectable by this assay. STAT92E-GFP DNA binding activity was only detectable upon stimulation by Hop (Fig. 3.13A, lane 3). In contrast, the STAT92E^{M647H}-GFP mutant yielded a clear band shift even under non-stimulated conditions (Fig. 3.13A, lane 6). This result indicated that this protein could constitutively bind DNA even in the absence of Hop stimulation. Moreover, this band migrated at the same position as the band shifted by STAT92E-GFP suggesting that STAT92E^{M647H}-GFP was also likely to bind DNA as a dimer like wild type STAT92E. Co-expression of STAT92E^{M647H}-GFP together with Hop increased the strength of the shifted band (Fig. 3.13A, lane 7). This was consistent with augmented Y704 phosphorylation of STAT92E^{M647H}-GFP after stimulation by Hop (see Fig. 3.12, compare A & B), indicative for a raised proportion of activated STAT92E. However, both STAT92E^{Y704F}-GFP and STAT92E^{M647H,Y704F}-GFP mutants did not show any DNA binding activity (Fig. 3.13A, lanes 4, 5 & 8, 9), whether stimulated or not. This again displayed the importance of Y704 for proper functioning of STAT92E-GFP.

Y704 phosphorylation of STAT92E-GFP was required to produce dimers capable of DNA binding. In addition, endogenous Hop activity was not detectable in the S2 cell line used. On this account, the question arose regarding the mechanism, by which STAT92E^{M647H}-GFP was phosphorylated. Inhibition of protein phosphatases is sufficient for phosphorylation and subsequent activation of STAT proteins independent of external pathway stimulation and JAK activity (Haque *et al.*, 1995). In an attempt to mimic Hop independent STAT92E^{M647H}-GFP phosphorylation, cultured cells expressing wild type STAT92E-GFP were treated with the phosphatase inhibitor sodium-ortho-vanadate (see Material & Methods 2.4.2). It was already pointed out that co-expression of STAT92E-GFP with Hop was sufficient to stimulate STAT92E-GFP DNA binding (see Fig. 3.13A). When non-stimulated S2 cells expressing STAT92E-GFP were treated with sodium-ortho-vanadate, a similar effect could be observed (Fig. 3.13B, lane 6). However, the intensity of the detected shifted band was not as strong as the intensity of the band caused by binding of STAT92E-GFP stimulated by Hop (Fig. 3.13B, compare lanes 5 & 6). Rather, it was similar to the one produced by non-stimulated

STAT92E^{M647H}-GFP (Fig. 3.13B, compare lanes 6 & 7). A possible explanation for this is that low levels of tyrosine kinase activity are endogenously present in S2 cells and capable of phosphorylating both alleles of STAT92E-GFP. Furthermore, these results suggested that normally the activity of endogenous protein phosphatases counteract this STAT92E phosphorylation under non-stimulated conditions. However, phosphatases were incapable of dephosphorylating STAT92E^{M647H}-GFP to the same degree as STAT92E-GFP.

When considered in combination, STAT92E^{M647H}-GFP fulfilled the characteristics expected of activated STAT92E-GFP following stimulation by Hop. Furthermore, the activity of STAT92E^{M647H}-GFP was dependent on the integrity and phosphorylation of the conserved tyrosine residue Y704. This suggested that both tyrosine phosphorylation and dimerization of STAT92E^{M647H}-GFP are reminiscent of normal STAT activation. As such, STAT92E^{M647H}-GFP represented a constitutive gain-of-function allele like the previously described STAT5A^{N642H}-Flag (Ariyoshi *et al.*, 2000) as judged by the three criteria of sub-cellular localization, Y704 phosphorylation and DNA binding.

3.2.5 Transcriptional activation by STAT92E-GFP variants

The key role of a transcription factor is the ability to induce target gene expression. STAT92E^{M647H}-GFP acted as a gain-of-function mutant as judged by the three criteria of sub-cellular localization, Y704 phosphorylation and DNA binding (see 3.2.2, 3.2.3 & 3.2.4).

Similar to mouse STAT5A^{N642H}-Flag (Ariyoshi *et al.*, 2000), STAT92E^{M647H}-GFP should be able to induce luciferase reporter gene expression in a JAK/STAT activity assay constitutively. Surprisingly, this was not the case. Expression of constitutively phosphorylated and DNA-bound STAT92E^{M647H}-GFP alone was not sufficient to induce expression of the luciferase reporter in cultured cells (Fig. 3.14, column 3, blue bar). Strikingly, even co-expression with Hop did not result in an increase in luciferase activity (Fig. 3.14, column 3, red bar). In contrast to STAT92E-GFP, STAT92E^{M647H}-GFP was incapable of activating transcription in this assay. STAT92E^{Y704F}-GFP was also not able to induce reporter gene expression (Fig. 3.14, column 4). Additionally, the double mutant STAT92E^{M647H,Y704F}-GFP was not capable of activating transcription after pathway stimulation (Fig. 3.14, column 5). Taken together, the integrity of Y704 was critical for JAK/STAT signaling.

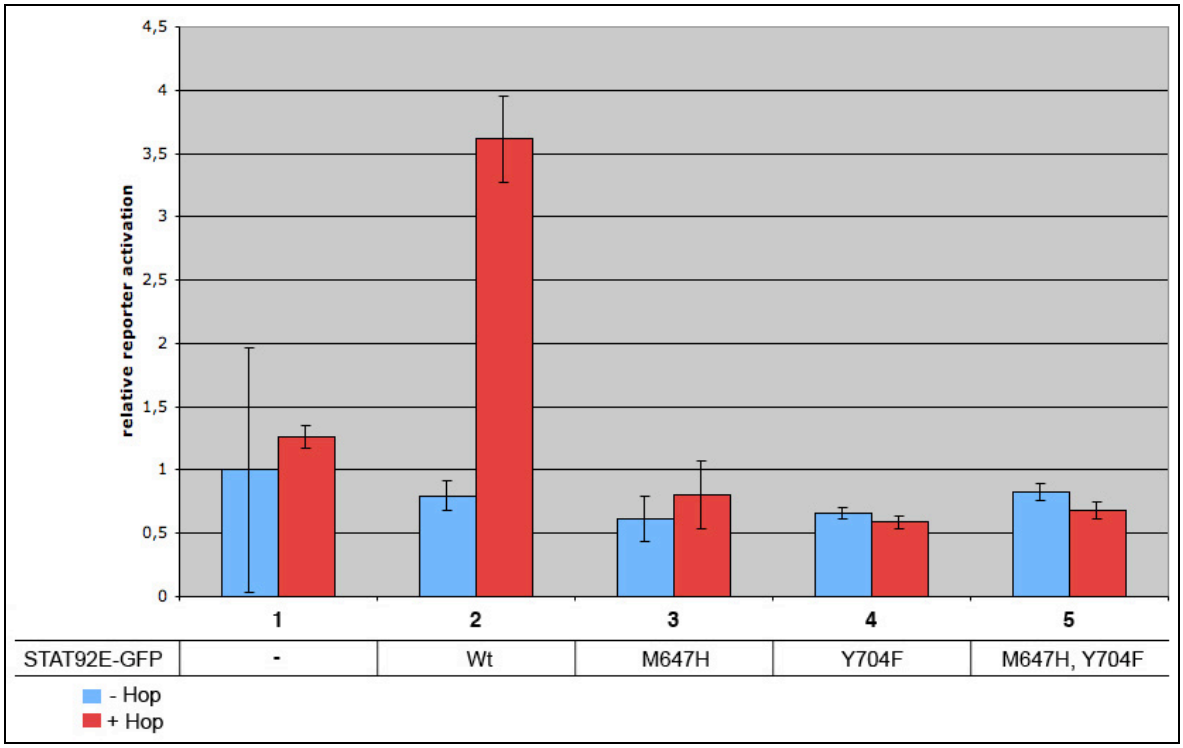

Fig. 3.14: **Transcriptional activation by STAT92E-GFP variants.** S2R+ cells (Yanagawa *et al.*, 1998) co-expressing the indicated proteins, the 2x*Draf*STATwt-TATA-luc reporter (Kwon *et al.*, 2000) and *Renilla* luciferase as normalization control. Lysates were used for dual luciferase reporter assays as described in Material & Methods 2.4.3.2. Relative fold activation (Firefly luciferase reporter activity / *Renilla* luciferase activity) of the luciferase reporter in lysates of cells not expressing Hop is shown in blue, relative activation in lysates of cells co-expressing Hop is shown in red. Mock-transfected cells were allocated the relative activity 1. Error bars represent standard deviations of four experiments. In contrast to wild type STAT92E-GFP (column 2), STAT92E-GFP mutant variants were not able to induce relative reporter activity both under non-stimulated and stimulated conditions (columns 3 - 5).

These results showed that STAT92E^{M647H}-GFP is not a constitutively active allele of STAT92E, as it was expected from its gain-of-function characteristics of constitutive phosphorylation (see 3.2.3), nuclear accumulation (see 3.2.2) and DNA binding (see 3.2.4). *Drosophila* STAT92E^{M647H}-GFP was not able to act as transcriptional activator in cell culture assays, thus functioning in a different way to mouse STAT5A^{N642H}-Flag (Ariyoshi *et al.*, 2000). Therefore, STAT92E^{M647H}-GFP function was analyzed *in vivo*. For this purpose, transgenic flies were generated, in which STAT92E-GFP variants could be expressed via the Gal4/UAS system (Brand and Perrimon, 1993).

3.2.6 Analysis of STAT92E-GFP variants *in vivo*

3.2.6.1 The JAK/STAT pathway during *Drosophila* eye development

JAK/STAT signaling is required for a range of developmental processes during the embryonic, larval and adult life of *Drosophila melanogaster* (reviewed in Zeidler *et al.*, 2000; Luo and Dearolf, 2001). This includes proliferation of cells within the eye imaginal disc, the larval tissue that gives rise to the adult compound eye (Bach *et al.*, 2003).

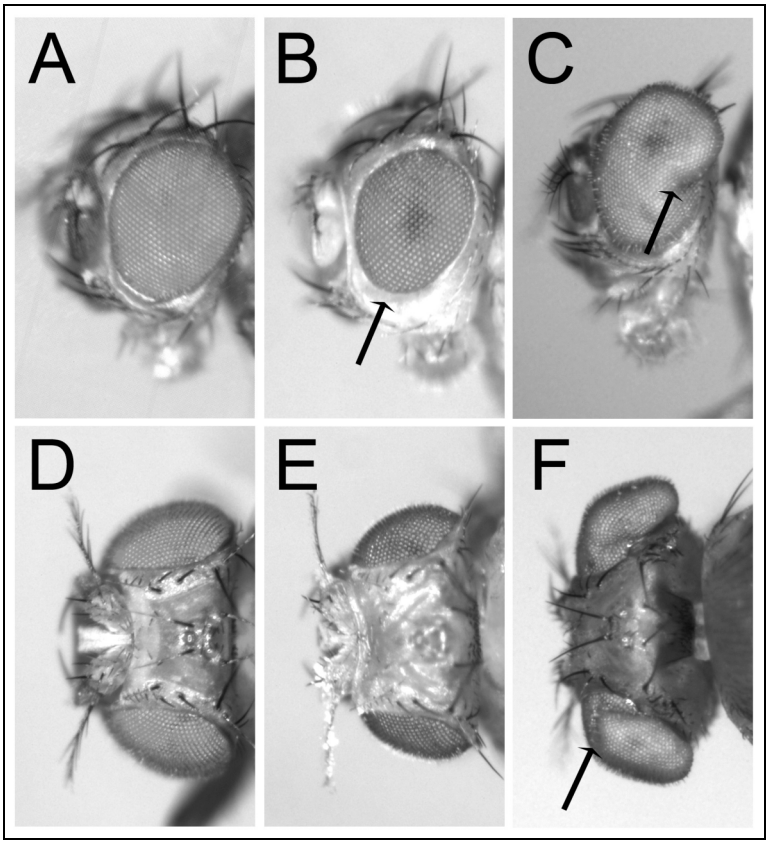

Fig. 3.15: **JAK/STAT signaling during eye development of *Drosophila*.** Eyes in (A) - (C) are shown in lateral view, eyes in (D) - (F) in dorsal view. **(A)** & **(D)** Eyes of an adult wild type fly. **(B)** & **(E)** Eyes of an adult fly mutant of Upd (allele Upd^{os1}) are smaller than wild type eyes (compare to A and D). Loss of ventral eye tissue is marked by an arrow in (B). **(C)** & **(F)** Ectopic expression of the pathway ligand Upd under the control of the GMR element in the developing eye resulted in overgrown adult eyes (Bach *et al.*, 2003), particularly observable in the dorsal region of the eye (arrows in C & F).

Loss of JAK/STAT activity during eye development resulted in a small-eye phenotype (Tsai and Sun, 2004) as shown in Fig. 3.15B & E for a fly mutant of the pathway ligand Upd (compare to wild type eye in Fig. 3.15A & D). Although the structure of the eye was not affected, a loss of ventral eye tissue was observable (arrow in Fig. 3.15B). Ectopic pathway

activation in the eye imaginal disc resulted in an overgrowth of the adult eye compared to the wild type eye (Fig. 3.15, compare A & C, see also 3.3.7), particularly observable in the dorsal regions of the eye (Bach *et al.*, 2003, arrows in Fig. 3.15C & F). Thus, changes in JAK/STAT pathway activity could be assayed on the basis of the variation of eye size and morphology. Therefore, STAT92E-GFP variants were expressed in the developing eye to assess their function *in vivo* in the context of *Drosophila* eye development.

Mis-expression of STAT92E-GFP variants during eye development

JAK/STAT components were expressed within the complete imaginal eye disc using the eye disc-specific driver line *eyeless-Gal4* (*ey-Gal4*) (Halder *et al.*, 1995). Expression of EGFP during eye development had no effect on either the size or the morphology of the resulting adult eye (Fig. 3.16A). However, expression of a dominant-negative allele of the pathway receptor Domeless lacking the cytoplasmic domain (DomeΔCyt, Brown *et al.*, 2001) resulted in a strong reduction of the overall volume of the eye and a loss of ventral tissue (arrow in Fig. 3.16B). Conversely, ectopic pathway activation by mis-expression of the constitutively active kinase Hop^{Tuml} caused overgrowth of the adult eye (Fig. 3.16C & D). This showed that modulation of JAK/STAT activity interferes efficiently with eye development of *Drosophila*. Although expression of STAT92E-GFP resulted in an occasional mild roughening of the posterior equatorial regions, no discernable change in eye size was observed as compared to the EGFP expressing eye (compare Fig. 3.16A & E). Similarly, expression of the non-functional $STAT92E^{Y704F}$-GFP did not have a visible effect on the eye morphology or size (Fig. 3.16G). By contrast, expression of $STAT92E^{M647H}$-GFP was sufficient to produce roughening and reduction of eye volume, readily identifiable by the loss of ventral eye tissue (arrow in Fig. 3.16F), an effect analogous to that produced by the dominant-negative DomeΔCyt (Fig. 3.16B). These results, confirmed by testing eight independent transgenic fly lines, suggested that mis-expression of $STAT92E^{M647H}$-GFP produced developmental defects consistent with its function as a dominant-negative allele *in vivo*. In the double mutant $STAT92E^{M647H,Y704F}$-GFP disruption of Y704 was sufficient to abrogate the phenotype produced by $STAT92E^{M647H}$-GFP (Fig. 3.16H), indicating that $STAT92E^{M647H}$-GFP required Y704 to exert the dominant-negative effect.

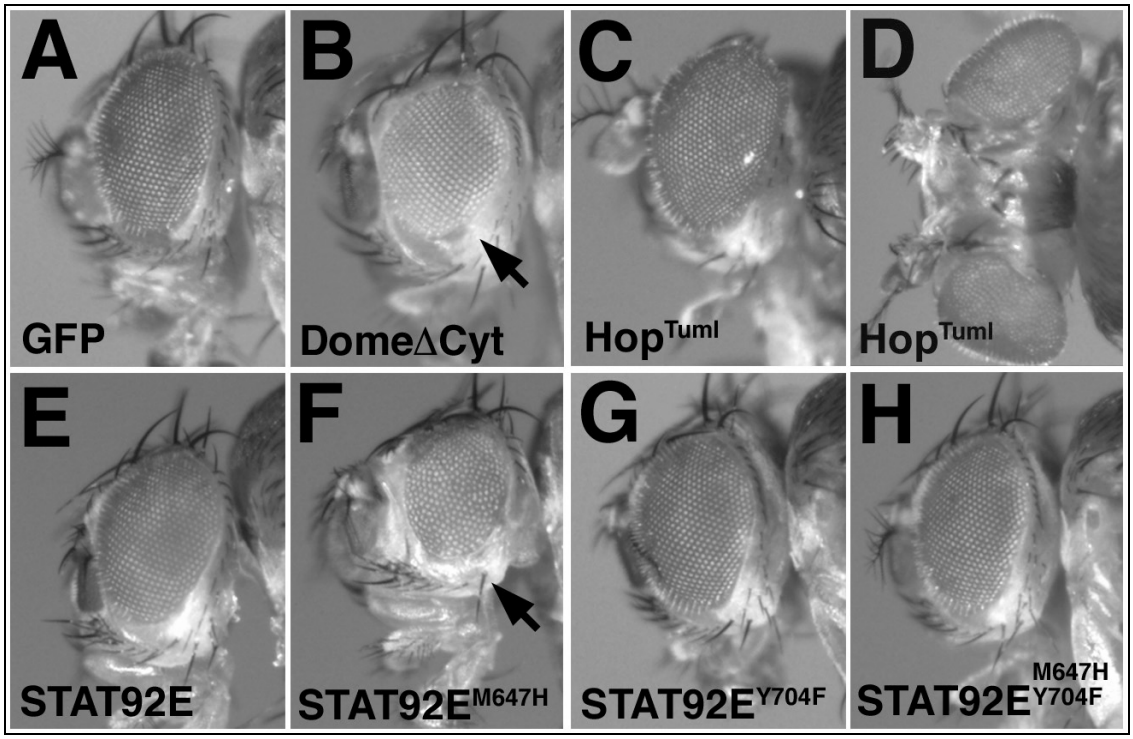

Fig. 3.16: **Effect of mis-expression of STAT92E-GFP variants on the developing *Drosophila* eye.** Adult eyes emanating from eye imaginal discs expressing the indicated control or STAT92E-GFP fusion proteins under the control of *ey-Gal4* (Halder *et al.*, 1995). Unless otherwise noted, all eyes are anterior to the left, dorsal up and show the lateral view. **(A)** Expression of GFP did not alter the size or morphology of the eye. **(B)** Expression of a dominant-negative receptor variant that lacks its cytoplasmic domain resulted in loss of ventral eye tissue (arrow) and a rough eye. **(C)** & **(D)** Expression of Hop^{Tuml} led to an overgrowth of the eye as shown in lateral (C) and dorsal view (D). **(E)** Expression of STAT92E-GFP had no effect on size or morphology of the eye. **(F)** Expression of $STAT92E^{M647H}$-GFP resulted in loss of ventral eye tissue (arrow) and a rough eye similar to the dominant-negative phenotype shown in (B). **(G)** & **(H)** Expression of $STAT92E^{Y704F}$-GFP (G) or $STAT92E^{M647H,Y704F}$-GFP (H) did not alter eye size or morphology.

3.2.6.2 The JAK/STAT pathway during *Drosophila* tracheal development

To test whether the mutation M647H affects other aspects of STAT92E activity besides cellular proliferation in the eye imaginal disc, the effect of $STAT92E^{M647H}$-GFP on expression of the JAK/STAT pathway target gene *trachealess* (*trh*) (Brown *et al.*, 2001) was analyzed. *trh* is one of the first markers of tracheal development in *Drosophila* and encodes a protein required for the development of the embryonic and larval tracheal system (Isaac and Andrew, 1996; Wilk *et al.*, 1996). Previous studies have shown that loss of Dome or STAT92E is sufficient to ablate *trh* expression (Brown *et al.*, 2001). Therefore, the expression of this gene was used as a molecular marker for JAK/STAT activity. STAT92E-GFP variants were

ubiquitously expressed during embryogenesis using the Gal4/UAS-system (Brand and Perrimon, 1993) and the *nullo-Gal4* line (Kunwar *et al.*, 2003).

The effect of mis-expression of STAT92E-GFP variants on the expression of the pathway target gene *trh*

In wild type embryos of stage 10/11 *trh* is expressed in the developing tracheal placodes (Fig. 3.17A; Isaac and Andrew, 1996; Wilk *et al.*, 1996). Ectopic expression of STAT92E-GFP did not affect *trh* expression (Fig. 3.17B). However, despite the presence of endogenous STAT92E activity in the developing tracheal placodes, mis-expression of STAT92E^{M647H}-GFP was sufficient to strongly reduce the levels of *trh* expressed (Fig. 3.17C). No tracheal pits formed in these embryos. This effect was very similar to that caused by the mis-expression of a dominant-negative splice from of STAT92E (ΔNSTAT92E) lacking its N-terminal domain (Fig. 3.17D; Henriksen *et al.*, 2002). These results indicated at the molecular level that STAT92E^{M647H}-GFP acted as a dominant-negative allele capable of preventing the function of endogenous STAT92E *in vivo*.

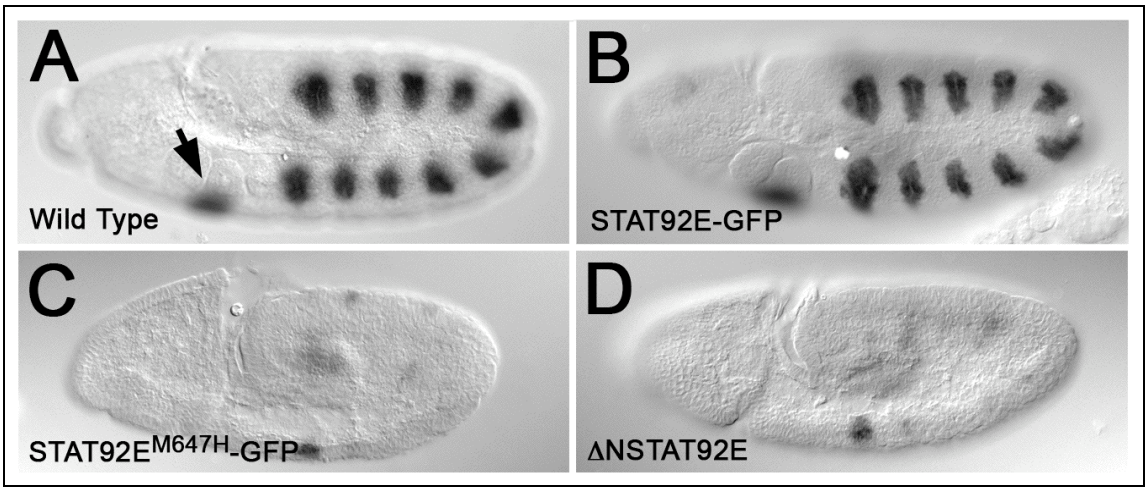

Fig. 3.17: **Effect of STAT92E-GFP variants on the expression of JAK/STAT pathway target gene *trh*.** *trh* RNA *in situ* hybridization using a DIG labeled antisense probe (see Material & Methods 2.8.1.2). Embryos are shown with their anterior to the left and dorsal up. Embryonic staging was carried out according to Campos-Ortega and Hartenstein (1997). **(A)** *trh* expression in stage 10/11 wild type embryos was strong and localized within the ten tracheal placodes and in the presumptive salivary gland primordia (arrow). **(B)** Expression of STAT92E-GFP in *w; P{w$^+$, Nullo-Gal4} / w; P{w$^+$,UAS-STAT92E-GFP}* embryos did not change the wild type pattern shown in (A). **(C)** By contrast, *trh* expression was almost completely ablated in *w; P{w$^+$, Nullo-Gal4} / w; P{w$^+$,UAS-STAT92E^{M647H}-GFP}* embryos of the same stage. **(D)** Expression of a dominant-negative form of STAT92E (Henriksen *et al.*, 2002) in *w; P{w$^+$, Nullo-Gal4} / w; P{w$^+$,UAS-ΔNSTAT92E}* embryos abolished *trh* expression in the tracheal placodes. Note that while expression of *trh* in the salivary gland

primordium does not require STAT92E (Brown *et al.*, 2001), ubiquitous mis-expression of dominant-negative STAT92E variants was sufficient to prevent expression in this tissue (C) & (D).

3.3 Involvement of other post-translational modifications of STAT92E in JAK/STAT signaling

In summary, the results described so far differed from the previous report, which identified the analogous mutant STAT5A^{N642H}-Flag as a constitutive active allele (Ariyoshi *et al.*, 2000). The mutation M647H of *Drosophila* STAT92E changed this protein into a dominant-negative allele *in vivo* (see Fig. 3.16 & Fig. 3.17). In contrast to mouse STAT5A^{N642H}-Flag (Ariyoshi *et al.*, 2000), expression of the *Drosophila* mutant protein was sufficient to prevent target gene expression (see Fig. 3.17). Consequently, the question arose why constitutively phosphorylated STAT92E^{M647H}-GFP was not able to activate target gene expression. The only difference between STAT92E-GFP and STAT92E^{M647H}-GFP is a single residue within the SH2 domain. In lower organisms like *C. elegans* a histidine is already incorporated at this position (see Fig. 3.10). Therefore, it appears very unlikely that this mutation was responsible for a complete loss of the ability to activate target gene expression. However, it also seems unlikely that a constitutively active STAT protein, whose activity is not controlled, exists in *C. elegans*. Therefore, a conceivable model could be that additional regulation of STAT activity aside from phosphorylation might be necessary for complete STAT activation.

3.3.1 CG9882, a protein with potential arginine methyltransferase activity

Dr. Steve Brown (Manchester University) identified a potential protein arginine methyltransferase (PRMT) annotated as CG9882 (FlyBase, 2003) as an interaction partner of both the receptor Dome and the kinase Hop in Yeast Two Hybrid (Y2H) assays (personal communication). Therefore, a potential role of arginine methylation in JAK/STAT signal transduction as additional regulatory mechanism was taken into consideration.

3.3.2 *CG9882* genomic organization and expression

The gene *CG9882*, identified in Y2H assays to interact with Dome and Hop, is located on the right arm of the 2nd chromosome at band 59C3. Its transcript is 2224 bases in length and

encodes a protein of 705 amino acids with predicted protein arginine methyltransferase activity (FlyBase, 2003). Its genomic organization is shown in Fig. 3.18.

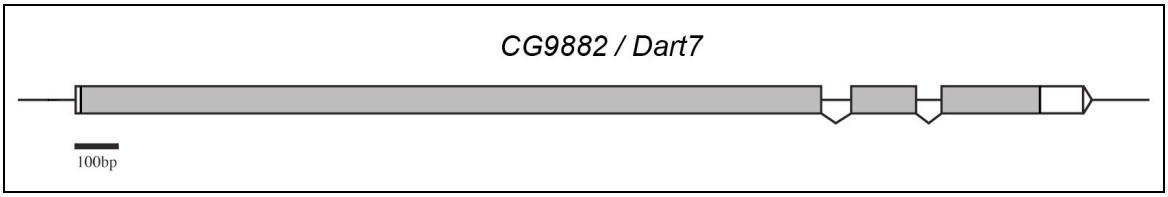

Fig. 3.18: **The genomic organization of the *CG9882* region.** Intron/exon structure for *CG9882/Dart7* is shown as predicted by the Berkeley *Drosophila* genome project (FlyBase, 2003). *CG9882/Dart7* consists of three exons and two introns. Coding sequences are indicated in grey, untranslated sequences in white. Scale bar represents 100bp.

To investigate a potential involvement of CG9882 in JAK/STAT signaling, its expression pattern was determined. During embryogenesis expression could not be detected by RNA *in situ* hybridization (data not shown). However, these results could not exclude a maternal protein contribution.

3.3.3 The role of arginine methylation in JAK/STAT signaling

Further evidence for an involvement of PRMTs in the JAK/STAT signaling cascade has been shown: PRMT1 binds to the cytoplasmic tail of the human IFNAR chain in the type I interferon receptor, which is involved in JAK/STAT signaling (Abramovich *et al.*, 1997). Moreover, PRMT1 associates with and methylates mouse STAT1 at a conserved arginine residue modulating IFNα/β-induced transcription (Mowen *et al.*, 2001). Arginine methylation has also been reported to be important for murine STAT6 function. Inhibition of methylation impaired phosphorylation and DNA binding activity of STAT6 (Chen *et al.*, 2004).
To assess the effect of arginine methylation on *Drosophila* JAK/STAT signal transduction, cell culture based experiments were performed.

3.3.4 Inhibition of methylation events by MTA

Protein methyltransferases can be inhibited by 5´-deoxy-5´(methylthio)adenosine (MTA) (Williams-Ashman *et al.*, 1982). It was reported that this inhibition did not affect transcription or translation in general, but had specific effects on JAK/STAT signaling (Mowen *et al.*, 2001). Therefore, MTA was used to inhibit methylation in cultured *Drosophila* cells (see

Material & Methods 2.4.2). The effect on JAK/STAT signaling was examined in a luciferase activity assay (Fig. 3.19). Effects on the general regulation of transcription were measured by co-transfection of a JAK/STAT-independent *Renilla* luciferase expression vector under the control of the *actin5.1* promoter.

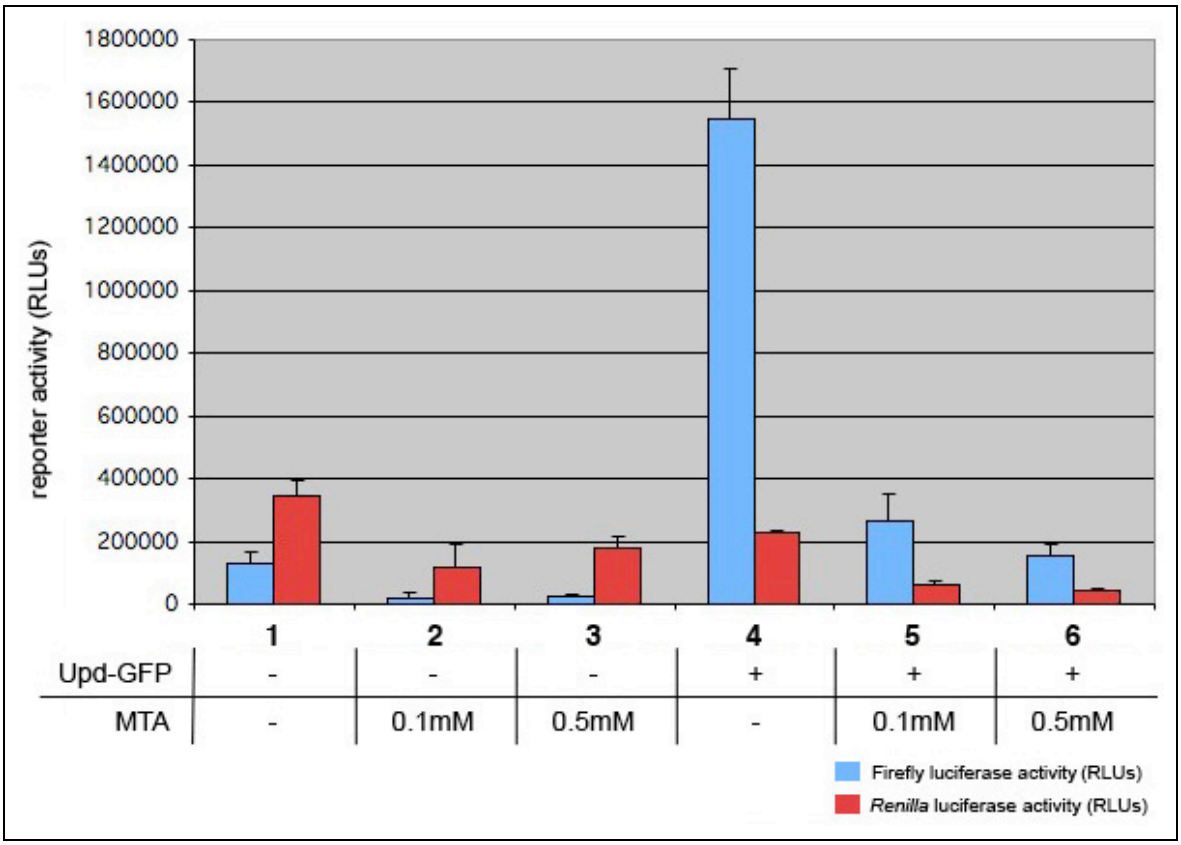

Fig. 3.19: **Effect of MTA on JAK/STAT signaling in cultured *Drosophila* cells.** S2 cells (Schneider, 1972) co-expressing the indicated proteins, the 2x*Draf*STATwt-TATA-luc reporter (Kwon *et al.*, 2000) and *Renilla* luciferase as control to assay JAK/STAT-independent effects of MTA. Lysates were used for dual luciferase reporter assays as described in Material & Methods 2.4.3.2. Cells were treated with the indicated concentration of MTA. Luciferase activity values (in RLU = relative light unit) of the same experiment are plotted. Firefly luciferase activity is shown in blue, *Renilla* luciferase activity in red. Error bars represent standard deviations of three experiments. MTA tratment of cultured cells adversely affected both JAK/STAT-dependent firefly and JAK/STAT-independent *Renilla* luciferase activities (columns 1 - 6).

Unlike the previous report, in which cells were treated with 0.3 mM MTA (Mowen *et al.*, 2001), an apparently non-specific effect on JAK/STAT signaling was observed. Under non-stimulated and stimulated conditions both luciferase activities were affected (Fig. 3.19). Firefly luciferase reporter activity decreased at increasing MTA concentrations (Fig. 3.19, blue bars). However, the JAK/STAT-independent *Renilla* luciferase activity also decreased

when cells were treated with increasing MTA concentrations (Fig. 3.19, red bars). These results suggested an overall non-specific effect of MTA on transcription or translation. If arginine methylation plays a role specifically in this signaling cascade, it is unverifiable in this approach because of the broadly effects caused by inhibition of methyltransferases.

3.3.5 BLAST search for *Drosophila* PRMTs

Apparently, methylation events affect multiple processes in a cell (reviewed in Chiang *et al.*, 1996), which makes it difficult to specifically uncover the role of arginine methylation in JAK/STAT signaling. To distinguish between effects of methylation on transcription or translation in general and a potential specific involvement in JAK/STAT signaling, I searched for putative PRMTs in *Drosophila* that may modulate JAK/STAT pathway activity using basic local alignment search tool (BLAST, Altschul *et al.*, 1990) searches of the *Drosophila* proteome. Mouse PRMT1 was reported to interact with the type I interferon receptor (Abramovich *et al.*, 1997) and to methylate STAT1 (Mowen *et al.*, 2001). Therefore, the sequence of this protein was used as query sequence for the BLAST search. Besides CG9882 eight other potential PRMTs were identified in the conceptual proteome of *Drosophila melanogaster* based on the genomic sequence release 3 (FlyBase, 2003). A search for protein domains in the InterPro database (Zdobnov and Apweiler, 2001) revealed that all of these proteins shared a SAM binding motif, which is essential for the function of methyltransferases. An alignment of these domains was performed using the clustal alignment algorithm of MegAlign software (DNASTAR Inc.; Higgins and Sharp, 1989) showing the similarity of this motif in the potential *Drosophila* PRMTs (Fig. 3.20A). Interestingly, comparison of the PRMT protein sequences revealed that CG9882 only showed about 10% sequence similarity to the other potential *Drosophila* PRMTs (Fig. 3.20B). However, the SAM binding motif of CG9882 showed a much higher similarity (about 33%) to the other PRMTs (Fig. 3.20C). *Drosophila* PRMTs were described recently (Boulanger *et al.*, 2004) and called *Drosophila* arginine methyltransferases (Dart). On the basis of its closest homolog PRMT7, CG9882 was called Dart7.

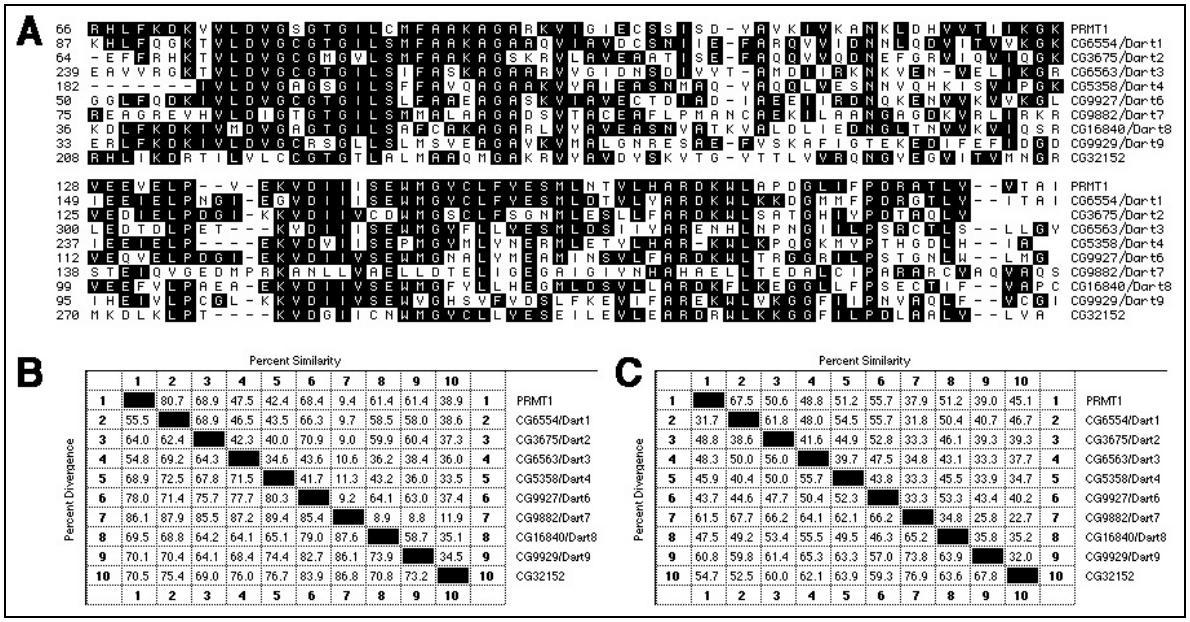

Fig. 3.20: **Potential *Drosophila* Protein Arginine Methyltransferases (Darts).** Identical amino acids are shaded black. Protein sequence identities are listed in Material & Methods 2.6. **(A)** Alignment of the predicted SAM binding motifs of mouse PRMT1 and potential Darts. **(B)** Calculated divergence and similarity of mouse PRMT1 and potential Darts (in %). **(C)** Calculated divergence and similarity of the SAM binding motifs shown in (A) of mouse PRMT1 and potential Darts (in %).

3.3.6 The role of Darts in JAK/STAT signaling in cultured cells

To examine the role of the identified predicted Darts in *Drosophila* JAK/STAT signaling, RNAi experiments were performed in the context of the luciferase reporter system (see Material & Methods 2.4.4). As shown in Fig. 3.9, it is possible to identify genes that interact with JAK/STAT signaling by using RNAi.

Cultured cells were transiently transfected with a construct that expresses Upd-GFP to activate JAK/STAT signaling. In these cells Darts were knocked down by treatment with dsRNA covering about 500bp of the ORF of the respective Darts. Expression of Upd-GFP resulted in an eight-fold induction of luciferase reporter activity (Fig. 3.21A, column 2). As expected, addition of *stat92E* dsRNA resulted in a reduction of the JAK/STAT-dependent reporter activity to a value, comparable to non-stimulated cells (Fig. 3.21A, column 3). Addition of dsRNA to specifically degrade *CG6554*/*Dart1*, *CG6563*/*Dart3*, *CG5358*/*Dart4*, *CG9929*/*Dart9* and *CG32152* mRNA did not have a significant effect on the relative luciferase reporter activity (Fig. 3.21A, columns 4, 6, 7, 11 & 12). Furthermore, simultaneous knockdown of all potential Darts only reduced JAK/STAT activity slightly (Fig. 3.21A, column 13). A similar effect was observed by knocking down the S-adenosylmethionine

synthetase (SamS) (Fig. 3.21A, column 14). SamS, also called M(2)21AB, catalyses the biosynthesis of SAM, the major methyl donor in methylation reactions (reviewed in Chiang *et al.*, 1996).

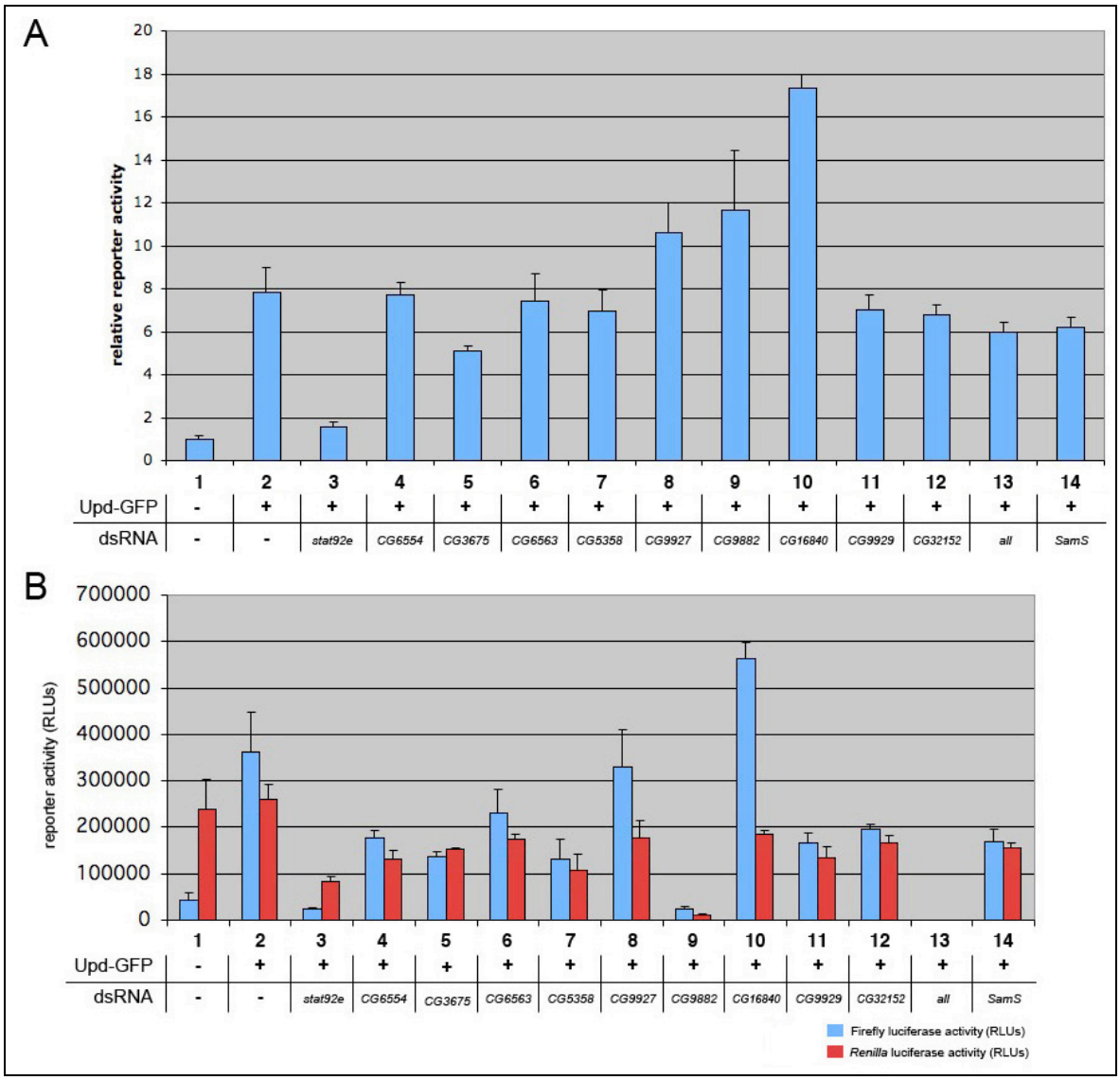

Fig. 3.21: **Effects of knocking down potential Darts by RNAi on JAK/STAT signaling in cultured cells.** S2 cells (Schneider, 1972) co-expressing the indicated proteins, the 2x*Draf*STATwt-TATA-luc reporter (Kwon *et al.*, 2000) and *Renilla* luciferase as normalization control. Cells were incubated with the indicated dsRNA (1.5 µg/10^5 cells) for three days until lysis. Lysates were used for dual luciferase reporter assays as described in Material & Methods 2.4.3.2. **(A)** Relative activity (Firefly luciferase reporter activity / *Renilla* luciferase activity) is plotted. Mock-transfected cells were allocated the relative activity 1. Error bars represent standard deviations of three experiments. Expression of Upd-GFP resulted in an eight-fold induction of luciferase reporter activity (column 2), which could be reduced to an almost non-stimulated level by addition of *stat92E* dsRNA (column 3). Treatment with *CG6554*, *CG6563*, *CG5358*, *CG9929* and *CG32152* dsRNA did not have an effect (columns 4, 6, 7, 11 & 12), whereas addition of *CG3675* dsRNA resulted in a decrease (column 5) and addition of *CG9927*, *CG9882* and *CG16840* dsRNA resulted in an increase of

relative reporter activity (columns 8 - 10). Simultaneous knockdown of all Darts or SamS only resulted in a slight decrease of relative reporter activity (columns 13 & 14). **(B)** Absolute luciferase activity values (in RLU = relative light unit) of the same experiment as shown in (A) are plotted. Firefly luciferase activity is shown in blue, *Renilla* luciferase activity in red. Error bars represent standard deviations of three experiments. Addition of *CG9882* dsRNA affected both JAK/STAT-dependent and -independent luciferase activities (column 9). Simultaneous knockdown of all Darts had a similar effect (column 13), whereas treatment with *SamS* dsRNA did not have a strong influence on luciferase activities (column 14).

Addition of dsRNA targeting sequence-specific for *CG3675*/*Dart2* resulted in a slight reduction of relative luciferase activity (Fig. 3.21A, column 5) suggesting a potential role as positive pathway regulator. Knockdown of *CG9927/Dart6* and *CG9882/Dart7* has a stimulating effect on relative luciferase reporter activity (Fig. 3.21A, columns 8 & 9). Therefore, a role as potential negative pathway regulator is possible. In comparison, addition of dsRNA to degrade *CG16840/Dart8* mRNA had a stronger stimulating effect on reporter activity. Inhibition of its expression doubled luciferase reporter activity from eight-fold to 16-fold (Fig. 3.21A, compare column 2 to 10). This indicated a stronger negative regulatory influence on JAK/STAT signaling than carried out by *CG9927*/*Dart6* or *CG9882/Dart7*.
However, a closer look at the absolute values of luciferase activities revealed that addition of *CG9882/Dart7* dsRNA caused a strong reduction in both firefly reporter and JAK/STAT-independent *Renilla* luciferase activity (Fig. 3.21B, column 9). This effect was aggravated when expression of all potential Darts was inhibited (Fig. 3.21B, column 13). Therefore, a general effect on transcription or translation caused by CG9882/Dart7 is more likely than a specific involvement in JAK/STAT signaling. Reducing expression levels of *SamS* should decrease the synthesis of the methyl donor SAM resulting in a lack of the substrate of Darts. As a consequence, methylation events were disturbed, which, in principal, should have a similar effect like simultaneously knocking down all Darts. However, knocking down the expression of *SamS* had only little effect on expression of both the firefly luciferase reporter and the *Renilla* luciferase control (Fig. 3.21B, column 14).

3.3.7 Interaction of arginine methylation and JAK/STAT signaling *in vivo*

The results of cell culture based assays described above suggested non-specific effects of arginine methylation on JAK/STAT signaling. At least, the apparently contradictory results of simultaneously knocking down all Darts and knocking down SamS made an interpretation of this data difficult. Therefore, the role of Darts and SamS in JAK/STAT signaling was

analyzed *in vivo*. For these studies mutants in the *Drosophila* PRMT1 homolog *Dart1*/*CG6554* and *SamS/M(2)21AB* were tested for their interaction with the JAK/STAT pathway. Unfortunately, no mutants for *Dart7*/*CG9882* or other *Darts* were available.

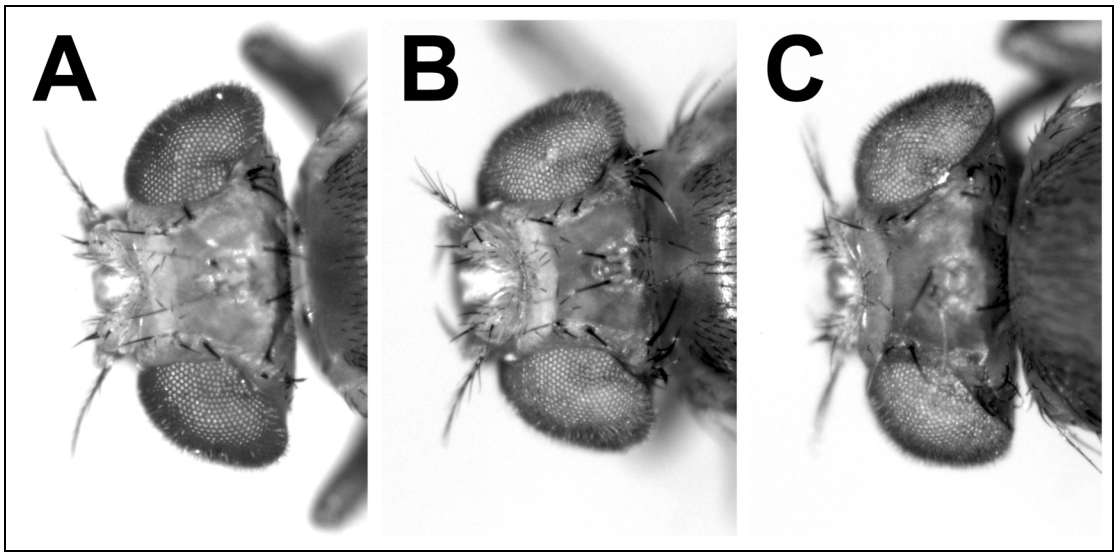

Fig. 3.22: **Interaction of JAK/STAT signaling with *Dart1 and SamS/M(2)21AB* in the developing *Drosophila* eye. (A)** *P{w*$^{+}$*, GMR-updΔ3´}*/+ eyes were overgrown, particularly on the dorsal side. **(B)** Removing one gene copy of the SAM synthetase *M(2)21AB* did not interact with ectopic Upd expression in the developing eye (*P{w*$^{+}$*, GMR-updΔ3´}*/+ ; *M(2)21AB*^{1}/+). **(C)** Removing one gene copy of *CG6554/Dart1* did not interact with ectopic Upd expression in the developing eye (*P{w*$^{+}$*, GMR-updΔ3´}*/+ ; *Art1*^{KG09631}/+).

The *Drosophila* eye was used as a model system to identify genetic interactions *in vivo* (see Material & Methods 2.7.3). To ectopically activate JAK/STAT signaling, the ligand Upd was expressed in the developing eye using the transgenic strain *P{w*$^{+}$*, GMR-updΔ3´}* (Bach *et al.*, 2003). In this transgene binding sites for the eye-specific transcription factor Glass (Ellis *et al.*, 1993) were multimerized and used for Upd expression posterior to the morphogenetic furrow in the eye imaginal disc. Upd expression by this Glass multimerized response (GMR) promoter ectopically activated the JAK/STAT pathway in the developing eye (see Fig. 3.15). This resulted in increased levels of cellular proliferation (Bach *et al.*, 2003), causing an overgrowth of the adult eye compared to the wild type eye, particularly observable in the dorsal regions of the eye (Bach *et al.*, 2003, see also Fig. 3.15). Removing positive regulators of the JAK/STAT pathway suppressed the eye overgrowth caused by Upd over-expression. Removal of one copy of *stat92E*, for example, counteracted the over-activation of the pathway and resulted in significant reduction in eye overgrowth (Mukherjee *et al.*, 2006).

However, neither suppression nor enhancement of the overgrown eye phenotype could be observed when a P-element insertion in the first exon of *CG6554* and the mutant *SamS* allele *M(2)21AB*^{1} were tested for genetic interaction with *P{w*$^{+}$*, GMR-updΔ3′}* (compare Fig. 3.22A with B & C). These results showed that the Upd dependent overgrowth phenotype is not sensitive to the gene dosage of *Dart1* and *SamS*. Additionally, this suggested that either methylation is not necessary for JAK/STAT signaling during eye development or that the methylation machinery is redundant with other components taking over the function of the affected genes. Furthermore, it is possible that one gene copy is still sufficient to produce enough protein activity to fulfill its entire function. A specific involvement of arginine methylation in JAK/STAT signaling thus remained unknown.

3.4 Identification of the potential target for methylation in STAT92E

Methylation affects a variety of different cellular processes (reviewed in Chiang *et al.*, 1996). Hence, inhibition of methylation events also affects innumerable processes in cells and thus is not an appropriate appliance to determine the role of methylation specifically in JAK/STAT signaling.

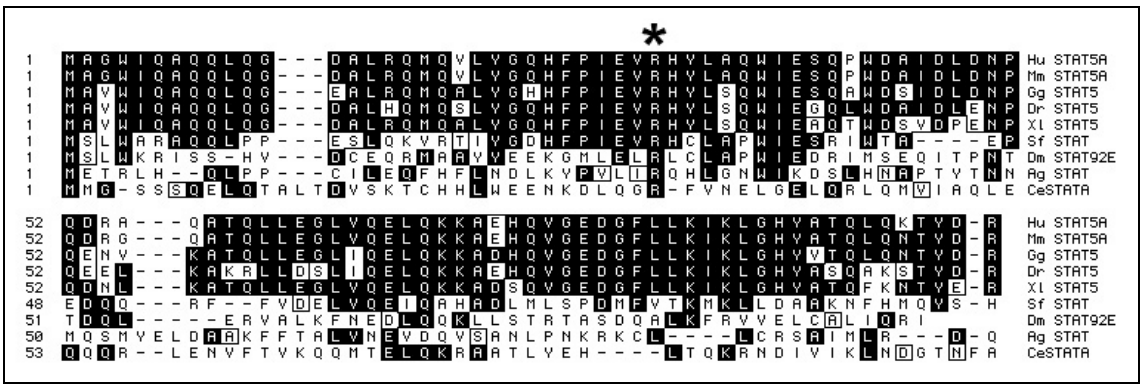

Fig. 3.23: **Alignment of the N-terminal region of several STAT proteins.** Identical amino acids are shaded black. Protein sequence identities are listed in Material & Methods 2.6. The asterisk marks the conserved arginine residue (R30 of *Drosophila* STAT92E). Hu = *Homo sapiens*, Mm = *Mus musculus*, Gg = *Gallus gallus*, Dr = *Danio rerio*, Xl = *Xenopus laevis*, Sf = *Spodoptera frugiperda*, Dm = *Drosophila melanogaster*, Ag = *Anopheles gambiae*, Ce = *Caenorhabditis elegans*.

To differentiate between the broad effects caused by inhibition of methylation in general and the specific involvement of arginine methylation in JAK/STAT signaling, the potential target residue for methylation of STAT92E was identified. It was reported that STAT1 was

methylated at an arginine residue conserved throughout the STAT family (Mowen *et al.*, 2001). Alignments of various STAT proteins using the clustal alignment algorithm of MegAlign software (DNASTAR Inc.; Higgins and Sharp, 1989) showed that in *Drosophila melanogaster* this residue within the N-terminal domain is an arginine at position 30 (R30). This residue is highly conserved in all STAT proteins (Fig. 3.23; Mowen *et al.*, 2001; and not shown). Analysis of STAT92E variants carrying a mutation in this residue should help to directly identify the influence of STAT methylation on signal transduction.

3.4.1 Generation and analysis of STAT92E^{R30mut}-GFP mutants

To examine the function of the conserved R30 and to directly affect its potential methylation, various STAT92E^{R30mut}-GFP mutants were generated on basis of previous reports (Mowen *et al.*, 2001; Komyod *et al.*, 2005). Arginine to alanine (R31A) and arginine to glutamic acid (R31E) substitutions were shown to increase the ability of mouse STAT1 to promote transcription by potentially mimicking arginine methylation (Mowen *et al.*, 2001). Therefore, the corresponding mutations R30A and R30E were introduced in *Drosophila* STAT92E-GFP. Additionally, R30 was substituted with lysine (R30K) to minimize potential structural effects on the N-terminal domain caused by R30 disruption (Komyod *et al.*, 2005). Furthermore, STAT92E^{R30mut,M647H}-GFP double mutants were generated to assess if a potential mutation mimicking methylation was sufficient to rescue the dominant-negative effect of STAT92E^{M647H}-GFP. This should be the case, if R30 methylation was missing for complete activation of STAT92E^{M647H}-GFP. R30mut, Y704F double mutants were also generated for negative control experiments as it was shown that Y704F mutation is sufficient to abolish STAT92E function (see Fig. 3.11 - Fig. 3.14).

3.4.2 Arginine methylation of STAT92E-GFP

Cell culture experiments were carried out to assess the methylation state of STAT92E-GFP. Whole cell lysates were examined by Western Blotting using an anti-Mono/Di-methyl-arginine antibody (see Material & Methods 2.5.2).

Although proteins that were arginine methylated were present in lysates of cells expressing STAT92E-GFP without or with Hop, only a faint band of the correct size was detectable (Fig. 3.24A, lanes 1 & 2, marked by dots). However, a protein of the same size could also be

detected in non-transfected cells used as negative control (Fig. 3.24A, lane 3, marked by dots). Therefore, this protein could not correspond to STAT92E-GFP.

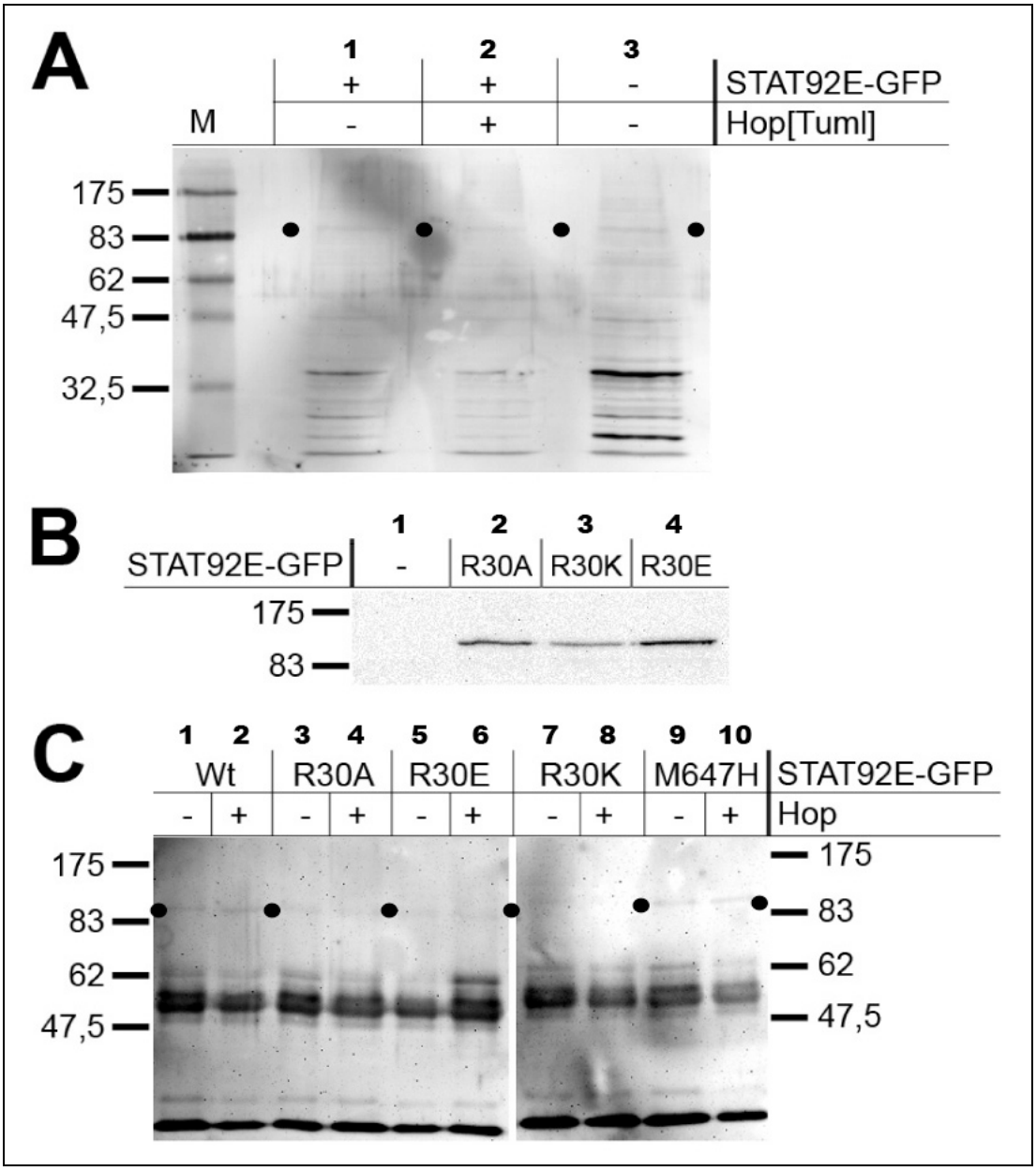

Fig. 3.24: Arginine methylation of STAT92E-GFP variants. (A) S2R+ cells (Yanagawa *et al.*, 1998) co-expressing the indicated proteins. Whole cell extracts were analyzed by Western Blotting using anti-Mono/Di-methyl-arginine antibody (see Material & Methods 2.5.2). In lysates of cells expressing STAT92E without or with Hop a band potentially corresponding to STAT92E-GFP was detected (lanes 1 & 2). However, this band was also detected in mock-transfected lysates (lane 3; respective bands are marked by dots). **(B)** Stability of $STAT92E^{R30mut}$-GFP variants. Lysates of S2R+ cells (Yanagawa *et al.*, 1998) expressing the $STAT92E^{R30mut}$-GFP variants were analyzed by Western Blotting using anti-GFP antibody. No degradation products could be detected indicating full length fusion proteins (lanes 2 - 4). **(C)** STAT92E-GFP was purified by IP using anti-GFP antibody (see Material & Methods 2.5.1) and analyzed by Western blotting using anti-Mono/Di-methyl-arginine antibody. A

band potentially corresponding to STAT92E-GFP was detected in purified lysates of cells expressing STAT92E-GFP or STAT92E^{M647H}-GFP (lanes 1 & 2, 9 &10). However, this band, albeit weaker, was also detected in purified STAT92E^{R30mut}-GFP samples (lanes 3 - 8; respective bands are marked by dots). Proteins of size between 47,5 and 62 kDa most likely corresponded to antibody polypeptide chains.

To assess possible differences between the methylation status of STAT92E-GFP and the mutated STAT92E-GFP variants, these proteins were expressed in cultured cells and purified from whole cell lysates in IP experiments with an anti-GFP antibody (see Material & Methods 2.5.1). First, STAT92E^{R30mut}-GFP variants were tested for their stability by Western blotting. No degradation products could be detected indicating full length and stable fusion proteins (Fig. 3.24B, lanes 2 - 4). After STAT92E-GFP IP, in immuno-precipitated lysates of cells expressing STAT92E-GFP or STAT92E^{M647H}-GFP a protein of the correct size could be detected by an anti-Mono/Di-methyl-arginine antibody (Fig. 3.24C, lanes 1, 2, 9 & 10, marked by dots). Yet, this band was also present, albeit weaker, in immuno-precipitated lysates containing STAT92E^{R30mut}-GFP variants (Fig. 3.24C, lanes 3 – 8, marked by dots) suggesting that either another arginine than R30 was methylated or that unspecific antibody binding has occurred. Repetitions of this experiment did also not lead to a conclusive result. In summary, arginine methylation of STAT92E-GFP could not be verified in these assays.
Although the results of the Western Blotting experiment did not indicate R30 methylation of STAT92E-GFP, the conservation of this residue among the STAT protein family suggested that this arginine held an essential role. Therefore, STAT92E^{R30mut}-GFP variants were expressed in cultured cells and *in vivo* and were functionally characterized. Examination of these mutant proteins included analysis of their sub-cellular localization, DNA binding ability, transcriptional activation and their effect *in vivo* as it was performed for STAT92E^{M647H}-GFP.

3.4.3 Sub-cellular localization of STAT92E^{R30mut}-GFP variants

STAT92E^{R30mut}-GFP mutant proteins were expressed in Kc_{167} cells (Cherbas *et al.*, 1977) and activated by co-expression of Hop^{Tuml}.

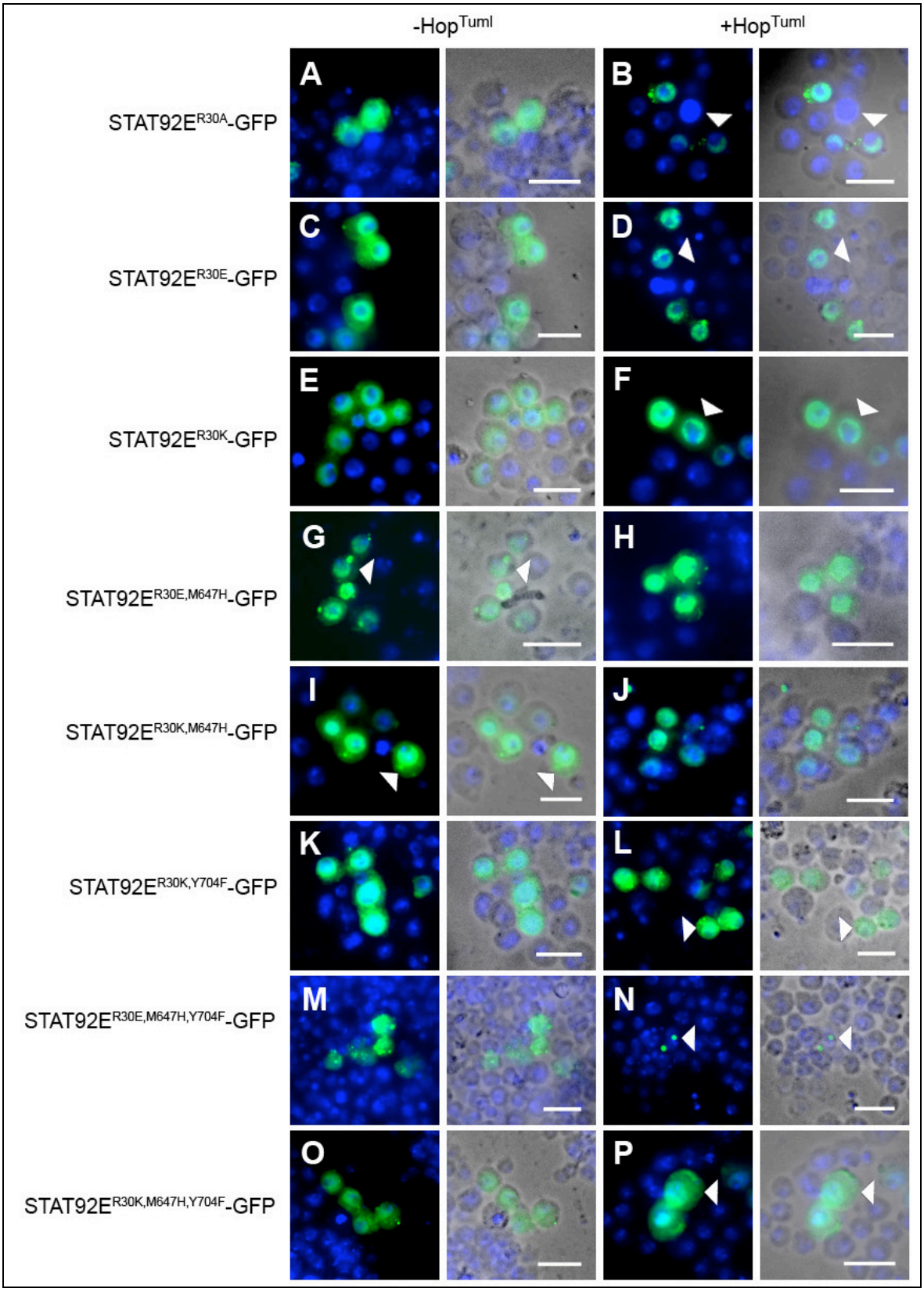

Fig. 3.25: **Sub-cellular localization of STAT92E^{R30mut}-GFP variants.** Kc_{167} cells (Cherbas *et al.*, 1977) co-expressing the indicated proteins. Columns 1 & 3 show GFP fluorescence and DAPI staining of DNA reflecting the localization of GFP fusion proteins (green) and the nuclei (blue), respectively. Columns 2 & 4 show columns 1 & 3 overlaid on a brightfield image of the cells. Scale bars represent 20 µM. **(A)**, **(C)** & **(E)** STAT92E^{R30mut}-GFP single

mutants are distributed throughout the cell including the nucleus under non-stimulated conditions. **(B)**, **(D)** & **(F)** STAT92E^{R30mut}-GFP single mutants accumulated in the nucleus upon stimulation by Hop^{Tuml} (arrows). **(G)** & **(I)** STAT92E^{R30mut,M647H}-GFP double mutants seemed to accumulate in the nucleus under non-stimulated conditions (arrows in G) with the exception of STAT92E^{R30K,M647H}-GFP, which is distributed throughout the cell (arrows in I). **(H)** & **(J)** Upon stimulation by Hop^{Tuml} STAT92E^{R30mut,M647H}-GFP double mutants accumulated in the nucleus. **(K)** & **(L)** STAT92E^{R30K,Y704F}-GFP double mutant was distributed throughout the cell under both non-stimulated (K) and stimulated (arrows in L) conditions. **(M)** - **(P)** STAT92E^{R30mut,M647H,Y704F}-GFP triple mutants were distributed throughout the cell under both non-stimulated (M & O) and stimulated (arrows in P) conditions. The STAT92E^{R30E,M647H,Y704F}-GFP triple mutant protein was presumably degraded upon stimulation with degradation products accumulating in the lysosome recognizable with GFP fluorescence in defined small areas (arrows in N).

All STAT92E^{R30mut}-GFP single mutants showed a behavior similar to STAT92E-GFP (see Fig. 3.5). Under non-stimulated conditions these variants were distributed throughout the cell including the nucleus (Fig. 3.25A, C & E). After stimulation with Hop^{Tuml} the fluorescence accumulated in the nucleus (arrows in Fig. 3.25B, D & F) indicating the translocation of STAT92E^{R30mut}-GFP variants from the cytoplasm into the nucleus. However, expression levels of STAT92E^{R30A}-GFP and STAT92E^{R30E}-GFP seemed to be reduced as compared to STAT92E^{R30K}-GFP or STAT92E-GFP as judged by the detectable amount of GFP signal. The double mutant proteins STAT92E^{R30A,M647H}-GFP and STAT92E^{R30E,M647H}-GFP seemed to accumulate in the nucleus even without stimulation by co-expressed Hop^{Tuml} (arrows in Fig. 3.25G). However, nuclear accumulation of non-stimulated STAT92E^{M647H}-GFP was more obvious (see Fig. 3.11). STAT92E^{R30K,M647H}-GFP did not accumulate in the nucleus under non-stimulated conditions (arrows in Fig. 3.25I). Nuclear accumulation was observable only after stimulation (Fig. 3.25J). Again, the R30K mutant showed the highest level of GFP fluorescence of the mutant proteins. Disruption of Y704 in the double mutant STAT92E^{R30K,Y704F}-GFP prevented nuclear accumulation (Fig. 3.25K & arrows in L). STAT92E$^{R30A/E,Y704F}$-GFP double mutant proteins appeared to be degraded after stimulation (data not shown). Triple mutant proteins STAT92E^{R30mut,M647H,Y704F}-GFP showed low level of GFP fluorescence and were distributed throughout the cell (Fig. 3.25M & O). Similar to the double mutants, R30A and R30E triple mutants seemed to be degraded after stimulation by co-expressed Hop^{Tuml} judged by the GFP signal in the cells. The degradation products presumably accumulated in the lysosome recognizable with GFP fluorescence within this cellular compartment (arrows in Fig. 3.25N). The R30K triple mutant protein was the most stable of the STAT92E-GFP variants as judged by detectable GFP fluorescence (arrows in Fig. 3.25P).

3.4.4 DNA binding ability of STAT92E^{R30mut}-GFP variants

STAT92E^{R30mut}-GFP mutant proteins were expressed in S2R+ cells (Yanagawa *et al.*, 1998) alone or together with Hop for JAK/STAT stimulation. Lysates generated from these cells were tested for the DNA binding activity by EMSA as described before (see 3.1.3.3).

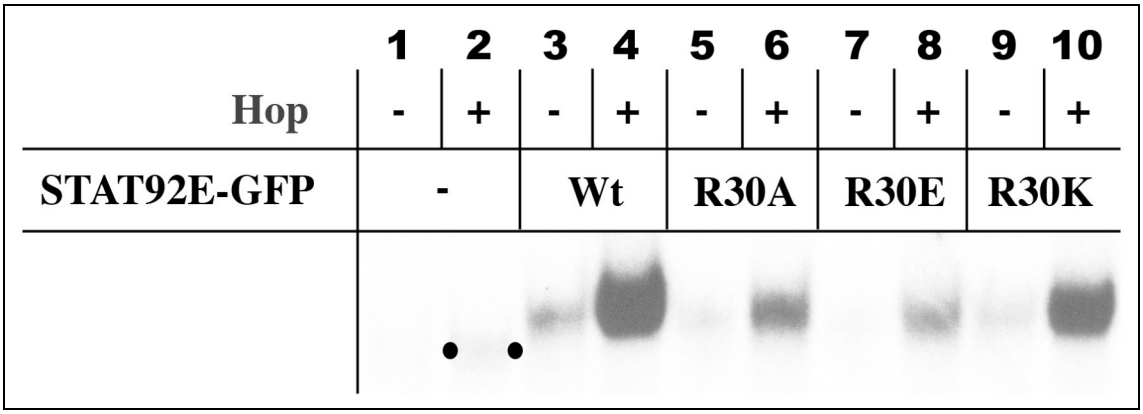

Fig. 3.26: **DNA binding ability of STAT92E^{R30mut}-GFP variants.** S2R+ cells (Yanagawa *et al.*, 1998) co-expressing the indicated proteins. Crude cell lysates were tested for DNA binding ability to radio-labeled oligonucleotides containing the STAT92E consensus DNA recognition site TTCCCGGAA (see Material & Methods 2.5.3). Expression of Hop alone presumably activated endogenous non-tagged STAT92E, recognizable with a faint band (lane 2, marked by dots) that ran faster than stimulated STAT92E-GFP (lane 4). Non-stimulated STAT92E^{R30mut}-GFP mutant proteins did not bind to DNA (lanes 5, 7 & 9). Co-expression with Hop (lanes 6, 8 & 10) resulted in a shifted band similar to that of activated STAT92E-GFP (lane 4).

No shifted bands were detectable in lysates of mock-transfected cells (Fig. 3.26, lane 1). However, in lysates of cells expressing Hop alone a faint shifted band was detectable (Fig. 3.26, lane 2, marked by dots), which ran faster than that produced by activated STAT92E-GFP (Fig. 3.26, lane 4). Most likely, this band corresponded to endogenous non-tagged STAT92E. Lysates of non-stimulated cells only expressing STAT92E^{R30mut}-GFP proteins did not display DNA binding activity (Fig. 3.26, lanes 5, 7 & 9), whereas co-expression of STAT92E^{R30mut}-GFP variants together with Hop resulted in a detectable DNA binding activity, visible by clear shifted bands (Fig. 3.26, lanes 6, 8 & 10). However, these mutant proteins bound significantly weaker than the wild type control (Fig. 3.26, compare lanes 6, 8 & 10 to lane 4), possibly due to their lower expression level (see 3.4.3). These results showed that STAT92E^{R30mut}-GFP mutants are capable of DNA binding upon activation by Hop, similar to STAT92E-GFP. Band shifts in non-stimulated lysates (e. g. non-stimulated STAT92E-GFP in lane 3) were only detectable in rare cases and were most likely non-

specific binding events due to protein over-expression using the Gal4/UAS system (Brand and Perrimon, 1993).

3.4.5 Transcriptional activation by STAT92E^{R30mut}-GFP variants

In principle, the STAT92E^{R30mut}-GFP single mutant proteins acted in a similar way as STAT92E-GFP, although differences in the expression level could be observed between the various arginine mutant proteins judged by the GFP fluorescence detected (see 3.4.3). R31A and R31E substitutions of mouse STAT1 were shown to increase signaling activity in a detectable amount under non-stimulated conditions (Mowen *et al.*, 2001). Therefore, homologous mutations of *Drosophila* STAT92E were tested in luciferase activity assays for the ability to elicit reporter gene expression (see Material & Methods 2.4.3.2).

The Kc_{167} cells used in this assay appeared to express endogenous STAT92E that can be activated by expression of the constitutive active kinase Hop^{Tuml}. Expression of Hop^{Tuml} alone resulted in approximately 4-fold induction of luciferase reporter activity (Fig. 3.27, column 1, red bar). STAT92E-GFP expression alone did not have an effect on reporter activity (Fig. 3.27, column 2, blue bar). Thus, endogenous pathway activity, able to activate STAT92E-GFP, was not present in these cells. Co-expression of STAT92E-GFP and Hop^{Tuml} resulted in an almost 25-fold increase in relative luciferase activity (Fig. 3.27, column 2, red bar). STAT92E^{R30mut}-GFP single mutants did not differ from the effect of STAT92E-GFP under non-stimulated conditions (Fig. 3.27, columns 3 - 5, blue bars). However, activation by Hop^{Tuml} only led to 8-fold induction of luciferase reporter activity (Fig. 3.27, columns 3 - 5, red bars). This was a higher level than the induction by Hop^{Tuml} alone indicating that STAT92E^{R30mut}-GFP mutant proteins were still able to induce reporter gene expression to some extent. However, in comparison to STAT92E-GFP this ability was strongly impaired. For STAT92E^{R30mut,M647H}-GFP double mutant proteins a similar effect could be observed (Fig. 3.27, columns 6 - 8). Mutant proteins, in which the conserved tyrosine residue Y704 was disrupted, did not show any transactivation activity that exceeded the reporter activation by Hop^{Tuml} alone, as shown for the double mutant STAT92E^{R30K,Y704F}-GFP and the triple mutant STAT92E^{R30K,M647H,Y704F}-GFP (Fig. 3.27, columns 9 & 10). The choice of the amino acid substituting the conserved R30 did not have a strong influence on the activity of the mutated STAT92E-GFP proteins, although R30K mutant proteins showed a slightly higher transcriptional activity than the other R30 mutants. However, I could not find evidence for

any of the STAT92E^{R30mut}-GFP variants acting as gain-of-function proteins as it was described for mouse STAT1 mutants in a previous report (Mowen *et al.*, 2001).

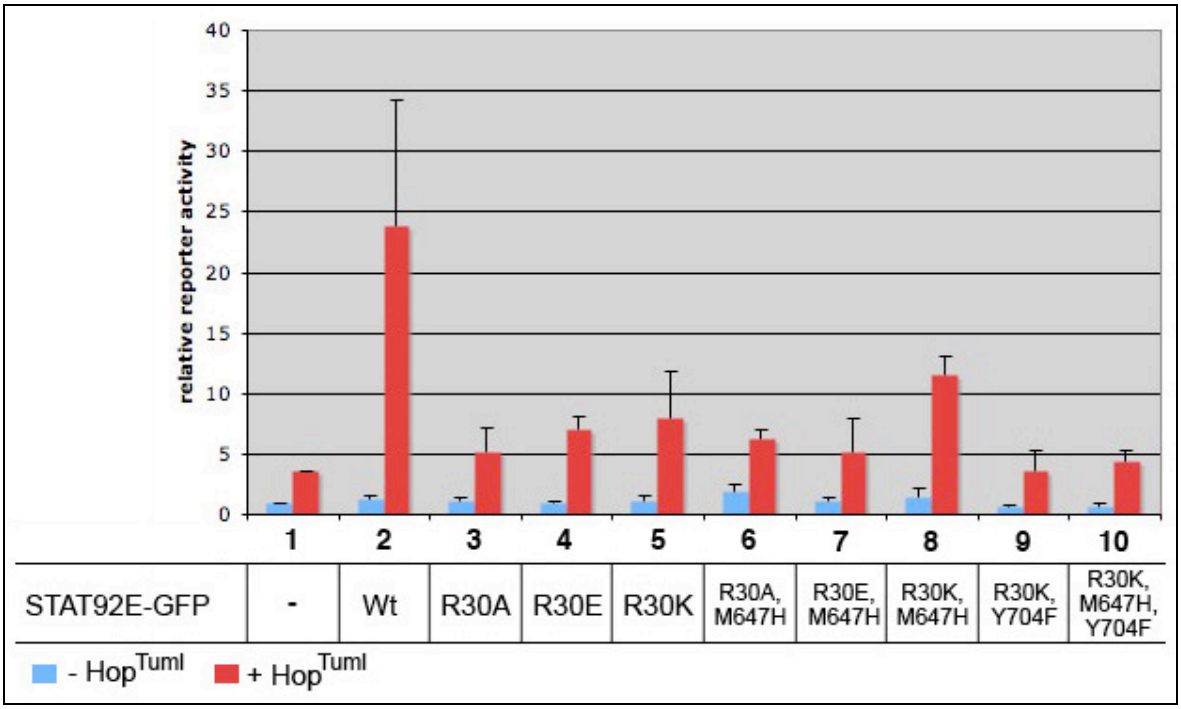

Fig. 3.27: **Transcriptional activation by STAT92E^{R30mut}-GFP variants.** Kc$_{167}$ cells (Cherbas *et al.*, 1977) co-expressing the indicated proteins, the 2x*Draf*STATwt-TATA-luc reporter (Kwon *et al.*, 2000) and *Renilla* luciferase as normalization control. Lysates were used for dual luciferase reporter assays as described in Material & Methods 2.4.3.2. Relative fold activation of the luciferase reporter (Firefly luciferase reporter activity / *Renilla* luciferase activity) in lysates of cells not expressing Hop^{Tuml} is shown in blue, relative activation in lysates of cells co-transfected with an expression construct for Hop^{Tuml} is shown in red. Mock-transfected cells were allocated the relative activity 1. Error bars represent standard deviations of six experiments. STAT92E^{R30mut}-GFP variants were not able to induce luciferase reporter gene expression to the same extent as STAT92E-GFP (compare column 2 to colums 3 - 10).

The results of the cell culture experiments described above suggested that STAT92E^{R30mut}-GFP mutant proteins were able to react properly on JAK/STAT activity by the criteria of sub-cellular localization and DNA binding, indicating proper Y704 phosphorylation. However, these mutants showed impaired transactivation activity in luciferase reporter assays (Fig. 3.27). To reveal the functional consequences of R30 substitutions *in vivo*, the effects of STAT92E^{R30mut}-GFP variants on the developing eye and on *trh* target gene expression were analyzed as described above (see 3.2.6.1 & 3.2.6.2).

3.4.6 Analysis of STAT92E^{R30mut}-GFP variants *in vivo*

3.4.6.1 Mis-expression of STAT92E^{R30mut}-GFP variants during eye development

To assess the effect of the STAT92E^{R30mut}-GFP variants on JAK/STAT signaling *in vivo*, the *Drosophila* eye was used as a model (see also 3.2.6.1). STAT92E^{R30mut}-GFP variants were expressed in the developing *Drosophila* eye using the *ey-Gal4* driver (Halder *et al.*, 1995).

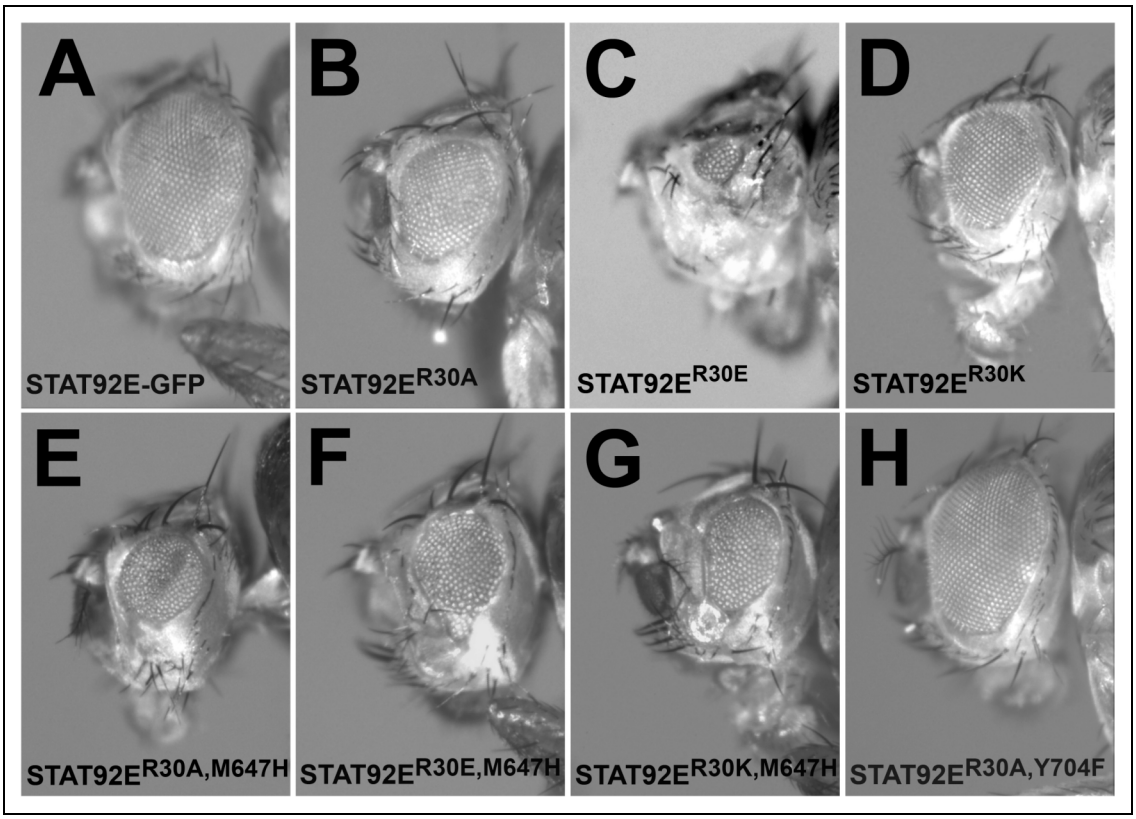

Fig. 3.28: **Effect of mis-expression of STAT92E^{R30mut}-GFP variants on the developing *Drosophila* eye.** Adult eyes emanating from eye imaginal discs expressing the indicated STAT92E-GFP fusion proteins under the control of *ey-Gal4* (Halder *et al.*, 1995). All eyes are anterior to the left and show the lateral view. **(A)** Expression of STAT92E-GFP did not alter the size or morphology of the eye. **(B)** Expression of STAT92E^{R30A}-GFP resulted in a small and rough eye. **(C)** Expression of STAT92E^{R30E}-GFP resulted in a small and rough eye, occasionally in an almost complete loss of the eye. **(D)** Expression of STAT92E^{R30K}-GFP led to a small and rough eye. **(E)** - **(F)** Expression of STAT92E^{R30mut,M647H}-GFP double mutants also resulted in a small and rough eye. **(H)** Expression of STAT92E^{R30A,Y704F}-GFP did not alter eye size or morphology.

Expression of STAT92E^{R30mut}-GFP single mutants in the developing eye produced a roughening and decrease of adult eye size (Fig. 3.28), similar to the dominant-negative alleles of the pathway receptor DomeΔCyt and STAT92E^{M647H}-GFP (see Fig. 3.16B & F). Expression of STAT92E^{R30A}-GFP and STAT92E^{R30K}-GFP in the eye imaginal disc resulted in

a consistent small and roughened eye phenotype (Fig. 3.28B & D). Expression of STAT92E^{R30E}-GFP resulted in phenotypes varying from a small reduction in eye size to an almost complete loss of an eye (Fig. 3.28C). Additionally, the eyes of the same fly could be affected to a different extent. Over-expression of STAT92E^{R30mut,M647H}-GFP double mutants produced a small eye phenotype, comparable to the STAT92E^{R30mut}-GFP single mutant phenotype (Fig. 3.28E - G). Disruption of Y704 resulted in the loss of the phenotype as shown for STAT92E^{R30A,Y704F}-GFP (Fig. 3.28H), suggesting that this residue and most likely its phosphorylation is important for STAT92E^{R30mut}-GFP mutants to exert their dominant-negative phenotype. These results were consistent in multiple independent transgenic lines.

3.4.6.2 The effect of mis-expression of STAT92E^{R30mut}-GFP variants on the expression of the pathway target gene *trh*

To assess the function of STAT92E^{R30mut}-GFP variants on a molecular level, the effect of these mutants on the expression of the JAK/STAT target gene *trh* (Isaac and Andrew, 1996; Wilk *et al.*, 1996) was analyzed (see also 3.2.6.2). For this purpose, STAT92E^{R30mut}-GFP variants were expressed ubiquitously in developing *Drosophila* embryos using the *nullo-Gal4* driver line (Kunwar *et al.*, 2003).

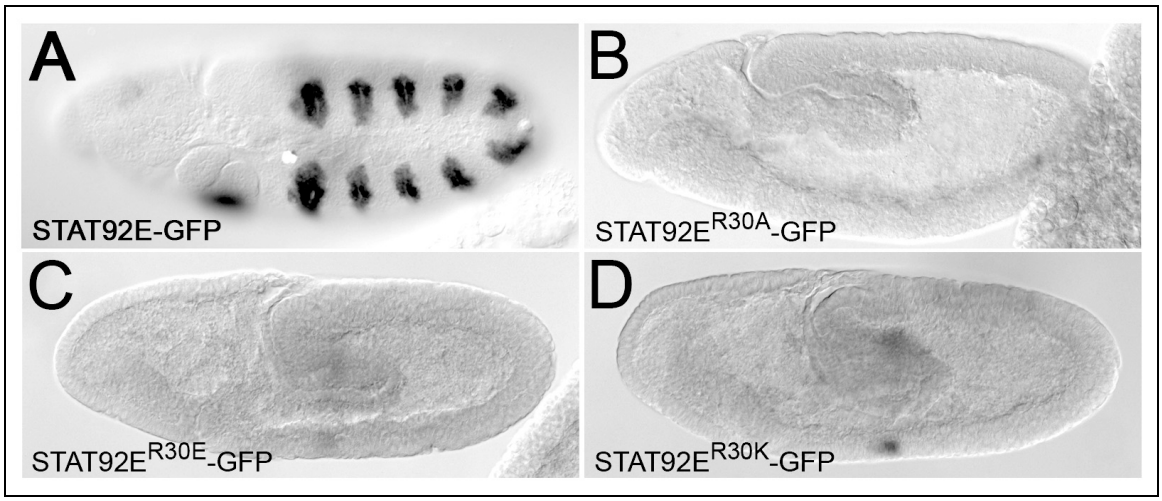

Fig. 3.29: **Effect of STAT92E^{R30mut}-GFP variants on the expression of JAK/STAT pathway target gene *trh*.** *trh* RNA *in situ* hybridization using a DIG labeled antisense probe (see Material & Methods 2.8.1.2). Embryonic staging was carried out according to Campos-Ortega and Hartenstein (1997). **(A)** At stages 10/11 *trh* expression in *w; P{w$^+$, Nullo-Gal4} / w; P{w$^+$,UAS-STAT92E-GFP}* embryos was strong and localized within the ten tracheal placodes and in the presumptive salivary glands. **(B)** – **(D)** By contrast, *trh* expression was almost completely ablated in *w; P{w$^+$, Nullo-Gal4} / w; P{w$^+$,UAS-STAT92E^{R30mut}-GFP}* embryos of the same stage. In (B) - (D) the salivary gland primordium was brought into focus

to show that ubiquitous mis-expression of dominant negative STAT92E^{R30mut}-GFP variants was also sufficient to prevent STAT92E independent *trh* expression in this tissue (Brown *et al.*, 2001).

As shown before, STAT92E-GFP did not affect the expression of the JAK/STAT target gene *trh* (Fig. 3.29A). However, expression of each of the three different STAT92E^{R30mut}-GFP single mutant proteins during embryogenesis was sufficient to strongly reduce *trh* expression levels (Fig. 3.29B - D). This indicated that STAT92E^{R30mut}-GFP variants act as dominant-negative alleles of STAT92E-GFP *in vivo*, similar to STAT92E^{M647H}-GFP and ΔNSTAT92E (see Fig. 3.17).

4 Discussion

JAK/STAT signaling is involved in many different developmental processes including hematopoiesis and the immune response (reviewed in O'Shea *et al.*, 2002). A wide variety of ligands are known in mammals to activate this signaling cascade by binding to their respective receptors (reviewed in Kisseleva *et al.*, 2002). Four JAK proteins (reviewed in Yamaoka *et al.*, 2004) transduce the signal via phosphorylation of a conserved tyrosine residue of seven STAT proteins (reviewed in Brierley and Fish, 2005). Phosphorylated STAT proteins dimerize, translocate to the nucleus and act as transcription factors by binding to specific sites in enhancers of their target genes and by subsequently activating their transcription (reviewed in Brierley and Fish, 2005). Specific combinations of receptors and JAK proteins ensure signal transduction through specific STAT dimers. As such, an external signal is transduced from the outside of a cell across the membrane, through the cytoplasm and finally into the nucleus.

In *Drosophila*, only three ligands (Unpaired (Upd) 1 - 3), one receptor (Domeless (Dome)), one JAK kinase (Hopscotch (Hop)) and one STAT protein (STAT92E) are known so far. Due to the lower complexity and redundancy of the pathway, *Drosophila melanogaster* is an excellent model organism for the analysis of the detailed mode of JAK/STAT signal transduction. Additionally, *Drosophila* is a genetic model organism with a long history. In combination with modern techniques it can be used as a powerful tool to investigate biological pathways *in vivo*. Classical genetic approaches to test gene interactions allow the identification of genes cooperating in a particular process. Furthermore, the relatively easy way of generating transgenic flies (Rubin and Spradling, 1982) in combination with the Gal4/UAS-system (Brand and Perrimon, 1993) allows the expression of a transgene in many different tissues and time points. This enables the analysis of mis-expression effects of mutant proteins *in vivo*. In this study, *Drosophila* STAT92E and mutant variants of STAT92E have been analyzed for their function in JAK/STAT signaling using cell culture and *in vivo* assays.

4.1 Tools for the analysis of *Drosophila* JAK/STAT activity

To visualize and to analyze *Drosophila* JAK/STAT activity in general and the function of STAT92E variants in particular, cell culture and *in vivo* assays were established.

4.1.1 A reporter for JAK/STAT activity

4.1.1.1 Expression pattern of *socs36e-lacZ* constructs

Activation of JAK/STAT signaling results in the change of expression of multiple genes. In *Drosophila socs36E* is one of these target genes (Callus and Mathey-Prevot, 2002; Karsten *et al.*, 2002). In the genomic region of *socs36E* 22 potential STAT92E DNA binding sites were identified (see Fig. 3.2; Karsten *et al.*, 2002), which all share the consensus recognition sequence TTCNNNGAA (Yan *et al.*, 1996). However, it was not known which binding sites are occupied *in viv*o by STAT92E dimers. To determine the minimal region containing all regulatory sequences for *socs36E* expression *in vivo*, *lacZ* reporter constructs including four different genomic regions with three to five clustered putative STAT92E binding sites (see Fig. 3.2) were engineered. These constructs were shown to be sufficient to activate *lacZ* reporter gene expression in patterns almost completely matching endogenous *socs36E* expression (see Fig. 3.3). Only very early aspects of the *socs36E* expression pattern, namely during stages 5 to 7 of embryogenesis, could not be reproduced. It is possible that under wild type conditions STAT92E dimers are bound to recognition sites of distinct enhancer regions. Interaction of these dimers might be required to produce the complete *socs36E* expression pattern. Cooperative binding of two STAT5 dimers to neighboring recognititon sites as tetramers, for example, is important for interleukin-2 induced target gene expression (Meyer *et al.*, 1997). In transgenic *Drosophila* embryos potential interactions between STAT92E dimers, bound in genomic regions that were separated by their cloning into the *lacZ* reporter construct, would have been disrupted and thus adversely affect reporter gene expression. In addition, binding sites of unknown STAT92E co-factors might have been also affected, thus interfering with the recapitulation of the endogenous *socs36E* expression pattern.

4.1.1.2 Luciferase reporter

The *lacZ* reporter construct including region I, which contains 5 putative STAT92E binding sites (see Fig. 3.2), was shown to be capable of activating *lacZ* reporter gene expression *in vivo*. The expression pattern particularly recapitulated the aspects of endogenous *socs36E* expression from stage 9 of embryogenesis (see Fig. 3.3). Furthermore, a bioinformatics survey concerning the number and spacing of the putative binding sites within the genomic region of *socs36E* resulted in a high probability for the sites of region I to contribute to endogenous *socs36E* expression *in vivo* (Ho-Ryun Chung, personal communication).

Therefore, a luciferase reporter construct based on region I was designed to monitor JAK/STAT activity in cell culture assays. Surprisingly, it was not possible to detect pathway activity in S2 cells using this reporter construct (see Fig. 3.4). S2 cells are derived from *Drosophila* embryos in late embryogenesis and are thought to represent a haemocyte- or macrophage-like cell type (Schneider, 1972). However, *in vivo lacZ* reporter expression was not detectable in haemocyte- or macrophage-like cells, but present in ectodermal tissues like the fore- and hindgut and the tracheal system (see Fig. 3.3). It is therefore possible that the luciferase reporter based on region I was not able to respond to STAT92E activity in S2 cells, because other tissue-specific or STAT independent control mechanisms were missing in these cells. However, using the reporter 2x*Draf*STATwt-TATA-luc, which contains two STAT92E binding sites from the promoter of the JAK/STAT target gene *Draf*, JAK/STAT activity was detectable in S2 cells (see Fig. 3.4; Kwon *et al.*, 2000). Therefore, this reporter and its modified version *6x2xDrafLuc* (Müller *et al.*, 2005) were used for further experiments.

4.1.2 Characteristics of STAT92E activation

To visualize the sub-cellular localization of *Drosophila* STAT92E and to analyze STAT92E function in general, a fluorescently tagged STAT92E fusion protein (STAT92E-GFP) was generated. Upon activation of the JAK/STAT cascade, this fusion protein was phosphorylated on a highly conserved tyrosine residue at position 704 (Y704) by the tyrosine kinase Hopscotch (Hop) as tyrosine phosphorylation was not detectable for STAT92E^{Y704F}-GFP mutants (see Fig. 3.6 & Fig. 3.12). Several other findings also suggest that the GFP fusion protein is really functional. It could be shown that activated STAT92E-GFP accumulates in the nucleus (see Fig. 3.5), binds a consensus STAT92E recognition site in an EMSA (see Fig. 3.7) and is able to induce luciferase reporter gene expression in cultured cells (see Fig. 3.8). However, over-expression of STAT92E-GFP during *Drosophila* development did not have an effect on JAK/STAT activity. On neither eye development (see Fig. 3.16) nor JAK/STAT target gene expression (see Fig. 3.17) a visible effect could be observed. Thus, elevated expression levels were not sufficient for an induction or inhibition of pathway activity in *Drosophila*. In contrast, it was shown that over-expression of a wild type STAT5 fusion protein in transgenic mice results in tumor growth similar to the one produced by over-expression of constitutively active STAT5 (Iavnilovitch *et al.*, 2004). This fact revealed potential functional differences between STAT proteins in various organisms and has to be considered when results of this study are discussed.

Taken together, it could be demonstrated that the STAT92E-GFP fusion protein is completely functional in cell culture assays in terms of sub-cellular localization, DNA binding ability and transcriptional activation of a luciferase reporter gene. Therefore, it was possible to use STAT92E-GFP in further assays to test STAT92E function.

Analysis of the characteristics in cultured cells and *in vivo* as described above provided a potent tool to analyze STAT92E mutants at different steps in their function. In addition, RNAi screens using luciferase reporter assays (see Fig. 3.9; Baeg *et al.*, 2005; Müller *et al.*, 2005) and assays for genetic interaction (for example, based on size alteration of the *Drosophila* eye (see Fig. 3.16; Bach *et al.*, 2003)) helped to characterize STAT92E variants and to identify components modulating JAK/STAT activity.

4.2 Functional analysis of STAT92E-GFP mutants

4.2.1 STAT92E^{M647H}-GFP

Constitutive activation of JAK/STAT signaling, often caused by gain-of-function mutations in JAK proteins, is implicated in the onset of several severe human diseases (reviewed in Schindler, 2002; Valentino and Pierre, 2006). Recently, the first disease-associated mutation in a STAT protein has been identified (Rosenfeld *et al.*, 2005). A patient with a lack-of-function mutation in the SH2 domain of STAT5b displayed a combination of severe growth retardation and immunodeficiency caused by affected growth hormone and cytokine signaling (Rosenfeld *et al.*, 2005). Although no natural gain-of-function allele of STAT proteins has been identified yet, constitutively active STAT variants were generated and analyzed (Bromberg *et al.*, 1999; Ariyoshi *et al.*, 2000). Functional analysis of these mutant STAT variants and the effects and mechanisms of STAT activation independent of stimulation by upstream pathway components could give an insight into the function of STAT proteins. It was shown that introduction of cysteine residues in the SH2 domain of murine STAT3 causes the formation of a sulfhydryl bond between STAT3 monomers resulting in covalent dimerization. These mutant STAT3 dimers translocate to the nucleus, bind DNA and activate transcription (Bromberg *et al.*, 1999). Furthermore, a mutation in the SH2 domain of mouse STAT5A, in which the amino acid asparagine at position 642 (N642) was substituted with a histidine (STAT5A^{N642H}), showed similar gain-of-function characteristics (Ariyoshi *et al.*, 2000). This mutant STAT5A-Flag fusion protein was constitutively phosphorylated resulting in a DNA bound STAT5A dimer that was able to induce target gene expression and was

sufficient to confer autonomous cell growth on interleukin-3-dependent Ba/F3 cells (Ariyoshi *et al.*, 2000). In summary, these results showed that STAT phosphorylation, dimerization and DNA binding together is sufficient to mediate transcriptional activation.

In the present study, a methionine at position 647 (M647) within the SH2 domain of *Drosophila* STAT92E was identified as the residue corresponding to N642 of mouse STAT5A (see Fig. 3.10). The accordant substitution of M647 to a histidine (M647H) led to constitutive Y704 phosphorylation of STAT92E-GFP (see Fig. 3.12), which was sufficient to cause nuclear accumulation (see Fig. 3.11) and constitutive DNA binding (see Fig. 3.13) similar to STAT5A^{N642H}-Flag (Ariyoshi *et al.*, 2000). STAT92E-GFP expression alone in contrast was not sufficient for its phosphorylation, nuclear accumulation and DNA binding (see Fig. 3.6, Fig. 3.5 & Fig. 3.7). Thus, endogenous JAK/STAT activity was not detectable in these assays suggesting that activation of STAT92E^{M647H} -GFP under non-stimulated conditions was independent of specific Hop activity.

A possible explanation for this non-specific activation could be a balance of continuous STAT phosphorylation and dephosphorylation at low levels under non-stimulated conditions proposed in a recent report (Liddle *et al.*, 2006). According to this working hypothesis, inhibition of phosphatases results in an unbalance causing constitutive STAT phosphorylation. Most likely, a similar mechanism was also responsible for *Drosophila* STAT92E^{M647H}-GFP phosphorylation under non-stimulated conditions. This model is supported by the fact that inhibition of protein phosphatases in cultured *Drosophila* cells by sodium-ortho-vanadate was sufficient to induce DNA binding of STAT92E-GFP independent of JAK/STAT activity (see Fig. 3.13). The intensity of the detected shifted band of non-stimulated STAT92E^{M647H}-GFP was comparable to that of STAT92E-GFP treated with sodium-ortho-vanadate. This suggested a similar way of activation via low levels of kinase activity for STAT92E^{M647H}-GFP.

It was hypothesized that dephosphorylation of activated STAT1 occurs after its release from DNA and is necessary for nuclear export of STAT1 (Meyer *et al.*, 2003). Although whole cell extracts were used to assay the DNA binding activity of STAT92E^{M647H}-GFP, it is very likely that the independently identified nuclear accumulation of this mutant variant coincides with constitutive DNA binding. It is possible that its higher affinity to DNA can slow down or prevent dephosphorylation of STAT92E^{M647H}-GFP in the nucleus and its transport back into the cytoplasm. The enhanced DNA binding activity I observed for STAT92E^{M647H}-GFP (see Fig. 3.13) thus could also contribute to the persisting phosphorylation and nuclear accumulation of this mutant.

The conserved tyrosine residue is essential for other mutant STAT proteins to fulfill their constitutive active function (Ariyoshi *et al.*, 2000; Liddle *et al.*, 2006). My results also indicated that corresponding Y704 is necessary in the process of STAT92E^{M647H}-GFP activation. The double mutant STAT92E^{M647H,Y704F}-GFP could not be phosphorylated (see Fig. 3.12). As a result, the protein lost its ability to accumulate in the nucleus (see Fig. 3.11) and to bind to DNA (see Fig. 3.13). This emphasized the essential role of the Y704 for STAT92E^{M647H}-GFP to exert its gain-of-function phenotype. In general, the highly conserved tyrosine residue plays a key role for the activation of STAT proteins.

4.2.1.1 Structural aspects of the M647H mutation

Phosphatases were incapable of counteracting the phosphorylation of STAT92E^{M647H}-GFP (see 4.2.1) and STAT5A^{N642H}-Flag mutants (Ariyoshi *et al.*, 2000) under non-stimulated conditions. One explanation for this is that the histidine substitution in these STAT variants increases the affinity of the mutated SH2 domains for the phosphorylated tyrosine in the dimer partner. This scenario would be consistent with molecular modeling of STAT92E based on the known structures of homo-dimerized mouse STAT3 (Becker *et al.*, 1998). Such a model suggests that in both vertebrate STAT5A^{N642H}-Flag and *Drosophila* STAT92E^{M647H}-GFP residues within the SH2 domains were substituted that have the potential to physically interact with the phospho-tyrosine residue of the dimerized partner (Fig. 4.1). In this model, M647 of *Drosophila* STAT92E is located near the pocket that binds the phosphorylated tyrosine of the STAT92E dimer partner. Substitution of this methionine with histidine (M647H) increases the positive charge in this pocket, potentially resulting in an increased affinity of the SH2 domain for the negatively charged phosphorylated tyrosine residue (Pierre Montaville & Stefan Becker (MPIbpc, Göttingen), personal communication; Ariyoshi *et al.*, 2000). This way, binding between the phosphorylated tyrosine and the SH2 domain is strengthened. The resultant enhanced interaction between STAT dimer partners, in turn, could restrict the access for cytoplasmic phosphatases under non-stimulated conditions preventing dephosphorylation. Consequently, a pool of phosphorylated STAT92E^{M647H}-GFP would gradually accumulate in the cell, which would result in constitutive dimerization and DNA binding of STAT92E^{M647H}-GFP similar to STAT92E-GFP activation by phosphatase inhibitors (see Fig. 3.13).

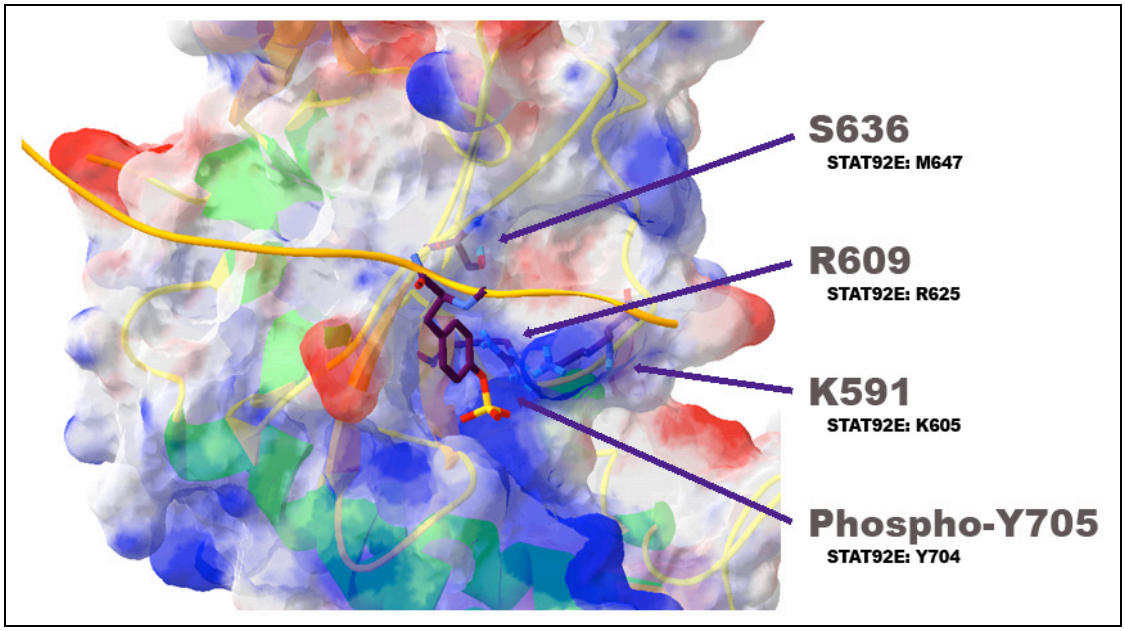

Fig. 4.1: Surface model of the phospho-tyrosine binding pocket of the STAT3 SH2 domain. Image kindly provided by P. Montaville (MPIbpc, Göttingen). Negatively charged residues are depicted in red, positively charged residues in blue. Corresponding residues of *Drosophila* STAT92E are listed below residues of mouse STAT3. The phosphorylated tyrosine is bound in a negatively charged pocket. Residue S636 of mouse STAT3 (or M647 of *Drosophila* STAT92E) is located near this binding pocket with the potential to interact with the phosphorylated tyrosine residue.

4.2.1.2 Transcriptional activation by STAT92E^{M647H}-GFP

Mouse STAT5A^{N642H}-Flag was reported to induce luciferase reporter and target gene expression independently of cytokine stimulation to a level equivalent to activated wild type STAT5A-Flag (Ariyoshi *et al.*, 2000). Therefore, the corresponding *Drosophila* mutant protein STAT92E^{M647H}-GFP was expected to permanently activate reporter gene expression. Surprisingly, this was not the case (see Fig. 3.14). Even co-expression of STAT92E^{M647H}-GFP together with Hop was not sufficient to induce luciferase reporter gene expression. In contrast to mouse STAT5A^{N642H}-Flag, constitutive phosphorylation of Y704 was not sufficient to completely activate *Drosophila* STAT92E^{M647H}-GFP in cultured cells. As proposed above (see 4.2.1), treatment of STAT92E-GFP with phosphatase inhibitors should mimic STAT92E^{M647H}-GFP activation by low levels of kinase activity under non-stimulated conditions. Therefore, it was tested whether phosphorylation of wild type STAT92E-GFP under such conditions is also insufficient for reporter gene expression. Unfortunately, it was not possible to address this question in a luciferase reporter assay. Short-time treatment of cultured cells with the phosphatase inhibitor was not sufficient for reporter gene expression to

an extent detectable in this assay. Prolonged treatment, however, resulted in cell death, making interpretations of such STAT92E activation impossible.

Taken together, these results suggested that STAT92E^{M647H}-GFP was phosphorylated by low levels of kinase activity. Its function under non-stimulated conditions appeared to be mimicked by STAT92E-GFP treated with phosphatase inhibitors. However, this phosphorylation, presumably occurring in the cytoplasm, was not sufficient for complete activation of STAT92E-GFP. Alternatively, it is also possible that the M647H mutation rendered STAT92E-GFP non-functional in terms of transcriptional activation while positively affecting its phosphorylation, sub-cellular localization and DNA binding.

4.2.1.3 STAT92E^{M647H}-GFP acts as a dominant-negative allele

Previous results identified the STAT5A^{N642H}-Flag mutant as a constitutively active allele of mouse STAT5A (Ariyoshi *et al.*, 2000) with the ability to confer autonomous cell growth on cytokine-dependent Ba/F3 cells. However, the proliferation rate was much slower as compared to cells that were treated with external cytokines. Additionally, levels of STAT5A target genes expression appeared to be similar or only modestly increased in cells expressing STAT5A^{N642H}-Flag as compared to cells expressing wild type STAT5A-Flag. However, autonomous growth of Ba/F3 cells expressing STAT5A^{N642H}-Flag did imply a gain-of-function effect for this mutant STAT protein.

The analysis of mutations in the context of an entire organism provides a potentially more accurate system than cultured cells, which often represent transformed cells differing in their behavior from wild type cells. In addition to cell culture assays, the function of the transactivational inactive *Drosophila* mutant protein corresponding to the constitutively active mouse STAT5A^{N642H}-Flag was therefore also analyzed *in vivo*. Expression of STAT92E^{M647H}-GFP in the developing *Drosophila* eye was sufficient to inhibit endogenous JAK/STAT activity, resulting in a small and rough eye phenotype. Expression of a dominant-negative form of the pathway receptor Domeless caused a similar phenotype (see Fig. 3.16). Additionally, expression of STAT92E^{M647H}-GFP during embryonic development was sufficient to prevent endogenous expression of the JAK/STAT pathway target gene *trh* (see Fig. 3.17). Taken together, STAT92E^{M647H}-GFP acted as a dominant-negative allele of STAT92E *in vivo*.

An explanation for the diverging behavior of the corresponding mutations of *Drosophila* and mouse STATs may be the redundancy of STAT proteins in mammalian cells.

Transcriptionally competent, but non-phosphorylated endogenous STAT proteins present in these cells may be able to hetero-dimerize with the constitutively phosphorylated STAT5A^{N642H}-Flag. These half-activated dimer complexes might be capable of inducing target gene expression at low levels, sufficient for the survival of the Ba/F3 cells. An explanation for the existence of such half-activated dimers might be provided in a recent report, in which non-phosphorylated STAT1 has been described to exist in an antiparallel dimeric structure (Zhong *et al.*, 2005). After phosphorylation a conformational rearrangement takes place and the SH2 domains bind the phosphorylated tyrosine of the respective dimer partner. In this model, half-activated hetero-dimers may form that possess the ability to activate target gene expression to a minor degree. The relative high expression levels in *Drosophila* cells that result from the Gal4/UAS system (Brand and Perrimon, 1993) increases the likelihood that STAT92E^{M647H}-GFP homo-dimers form. As such, a scenario in which transcriptionally incompetent STAT92E^{M647H}-GFP homo-dimers compete with half-activated hetero-dimers for STAT92E binding sites may represent the more accurate description of this particular mutation *in vivo*.

Additionally, it cannot be ruled out that different functions of *Drosophila* STAT92E and mouse STAT5A contribute to the diverging behavior of the respective mutant variants. Over-expression of a wild type STAT5 fusion protein was sufficient to induce tumor growth in mice, similar to a constitutively active chimeric STAT5 variant (Iavnilovitch *et al.*, 2004; see also 4.1.2). In contrast, no gain-of-function phenotype could be detected for *Drosophila* STAT92E-GFP over-expression (see Fig. 3.16).

Furthermore, it was reported that inactivation of STAT1 induced transcription appeared to be primarily governed by the dissociation rate of the STAT1-DNA complex, a frequency dictated by the particular recognition site and the affinity of STAT1 binding to this site (Meyer *et al.*, 2003). Most likely, STAT92E^{M647H}-GFP dimers competed against endogenous wild type STAT92E dimers for STAT recognition sites in enhancers of target genes. An enhanced binding of STAT92E^{M647H}-GFP to DNA might have changed the dissociation rate resulting in constitutive DNA binding (see Fig. 3.13) providing an advantage for STAT92E^{M647H}-GFP over wild type STAT92E. Hence, the access to these enhancer elements for endogenous STAT92E would be blocked. Taken together, the dominant-negative effect observed (see Fig. 3.16 & Fig. 3.17) was possibly based on DNA-bound, transcriptionally incompetent STAT92E^{M647H}-GFP dimers, preventing the expression of JAK/STAT pathway target genes.

In summary, Y704 phosphorylation was sufficient for translocation of STAT92E-GFP to the nucleus and its DNA binding (see Fig. 3.5 & Fig. 3.7). However, in the STAT92E^{M647H}-GFP mutant protein Y704 phosphorylation was not sufficient for the induction of JAK/STAT pathway target gene expression (see Fig. 3.17). This mutant represents the first STAT protein, for which tyrosine phosphorylation, nuclear accumulation and DNA binding is not sufficient for complete STAT activation. However, the mechanism, by which it exerts its dominant-negative phenotype, remains unclear.

Strikingly, in less complex organisms such as *C. elegans* a histidine is found in the position corresponding to N642 of mouse STAT5A or M647 of *Drosophila* STAT92E (see Fig. 3.10), respectively. Therefore, it is very unlikely that this mutation in SH2 domains alters these STAT proteins to be utterly non-functional. However, it is possible that STAT-like molecules of these organisms are constitutively phosphorylated and DNA-bound. Furthermore, no JAK-like molecules have been identified in the *C. elegans* genome (The *C. elegans* Sequencing Consortium, 1998). Therefore, it might be that STAT activity in *C. elegans* is controlled by additional mechanisms independent of tyrosine phosphorylation, conceivably via the modulation of transactivation activity. Thus, it appears possible that signaling via STAT is controlled on levels other than phosphorylation. In organisms where JAK proteins mainly impart pathway activation, this additional control mechanism could be necessary for full STAT activation. STAT92E^{M647H}-GFP might lack this control mechanism explaining the inability to be completely activated and to induce target gene expression.

4.3 Arginine methylation of STAT92E

As discussed above, STAT92E^{M647H}-GFP was constitutively phosphorylated, which was originally thought to be sufficient for complete activation of this protein. However, this STAT92E variant acted as a dominant-negative allele *in vivo*. The mechanism causing the dominant-negative phenotype remains to be elucidated. One possibility is that low level of kinase activities mediated constitutive STAT92E^{M647H}-GFP phosphorylation (see 4.2.1) in the cytoplasm. Yet, it is also possible that STAT92E have to receive another modification at the receptor/JAK complex for its complete activation. Data of Dr. Steve Brown (University of Manchester) support this hypothesis. He found a potential protein arginine methyltransferase (PRMT) bound to both the receptor Dome and the kinase Hop in Y2H screens. In addition, STAT1 arginine methylation was reported to modulate STAT activity by preventing interaction with the negative regulator PIAS (Mowen *et al.*, 2001). Furthermore, PRMT1

binds to the intracytoplasmic domain of the type I interferon receptor (Abramovich *et al.*, 1997) indicating a role for arginine methylation in JAK/STAT signaling. Moreover, treatment of cultured cells with 5´-deoxy-5´(methylthio)adenosine (MTA), an inhibitor of methyltransferases, was reported to specifically inhibit JAK/STAT signaling by reducing STAT1 arginine methylation (Mowen *et al.*, 2001). However, the specificity of this inhibition is unclear since another report demonstrated that MTA treatment adversely affects multiple biological processes (Williams-Ashman *et al.*, 1982). In *Drosophila* cell culture experiments MTA treatment also resulted in a rather non-specific effect in the dual luciferase reporter system, since both JAK/STAT-dependent firefly luciferase reporter and JAK/STAT-independent *Renilla* luciferase expression levels were affected (see Fig. 3.19). Meissner and colleagues obtained similar results showing a negative effect of MTA on STAT1 independent NF-κB reporter gene transcription (Meissner *et al.*, 2004). Because of the broad effects of MTA on various processes, including general transcription, specific effects of STAT92E methylation could not be addressed with those experiments. Therefore, determination of the role of STAT92E arginine methylation during JAK/STAT signaling required a more specific inhibitor than MTA.

4.3.1 *Drosophila* arginine methyltransferases

In order to circumvent the broad effects of MTA treatment, a more specific way to inhibit arginine methylation was applied. To do so, the *Drosophila* genome was searched for potential enzymes mediating protein arginine methylation. A BLAST search (Altschul *et al.*, 1990) for *Drosophila* protein arginine methyltransferases (Dart) identified nine candidate proteins. Typically for methyltransferases, all these proteins contain a domain for the binding of the main methyl donor SAM (see Fig. 3.20). CG9882/Dart7 identified in Y2H assays by Steve Brown (University of Manchester) was also among those candidate proteins. However, the closest homolog to PRMT1, which was shown to be involved in modulating mammalian JAK/STAT activity (Mowen *et al.*, 2001), was CG6554/Dart1. Actually, CG9882/Dart7 shares only low similarity to mouse PRMT1 and the other Darts (see Fig. 3.20).

Expression of *Darts* was detectable by Northern Blotting at various developmental stages suggesting that arginine methylation is essential during *Drosophila* development (Boulanger *et al.*, 2004). Consistently, RNAi experiments undertaken in the present study indicated a potential role for several Darts in JAK/STAT signal transduction (see Fig. 3.21). CG9927/Dart6 and CG16840/Dart8 acted as negative regulators of JAK/STAT signaling.

Knocking down their mRNA resulted in an increase of luciferase reporter activity. The opposite effect was observed for CG3675/Dart2 suggesting a role as positive regulator. CG6554/Dart1, the closest homolog to PRMT1, which was shown to be involved in JAK/STAT signaling (Abramovich *et al.*, 1997; Mowen *et al.*, 2001), did not interact with the JAK/STAT pathway in this assay. However, as indicated by Y2H assays, the most promising candidate for an involvement in JAK/STAT signaling was CG9882/Dart7, identified as interaction partner of Dome and Hop. When the expression of this gene was knocked down in the dual luciferase reporter assay system, both luciferase reporter and control activity were strongly reduced. This result indicated a non-specific effect rather than a specific involvement of CG9882/Dart7 in JAK/STAT signaling. However, it is possible that off-target effects caused by *CG9882/Dart7* dsRNA had an impact on these results, because knocking down the synthetase of the main methyl donor SamS to inhibit methylation in general surprisingly did not have a comparable effect. One possibility is that the *SamS* RNAi might have been inefficient, resulting in remaining SamS protein activity. Additionally, methylation of histones, for example, can have both positive and negative regulatory effects on transcription (Lee *et al.*, 2005). Thus, the generalized effects on methylation caused by knocking down *SamS* might camouflage specific effects on the reporter assay. Furthermore, CG9882/Dart7 might also affect other processes than arginine methylation explaining the differing effects observed for knocking down CG9882/Dart7 and SamS. Genetic interaction assays were performed to determine the role for methylation in JAK/STAT signaling *in vivo*. However, for neither a mutant allele of SamS nor a P-element insertion in *CG6554/Dart1* an interaction with JAK/STAT signaling during eye development was detectable (see Fig. 3.22). Unfortunately, no mutant alleles of *CG9882/Dart7* or other Darts were available. Thus, the effect of arginine methylation on JAK/STAT activity could not be readily addressed by these assays.

Although these results do not exclude the possibility of STAT methylation, it appeared more likely that protein methylation is involved in general transcriptional regulation. Arginine methylation of several proteins of the transcriptional machinery is required for protein-protein interactions regulating transcriptional initiation (Stallcup *et al.*, 2003). Inhibition of these modification events could affect these interactions resulting in a negative effect on transcriptional activation. The protein arginine methyltransferase CARM1, for example, was shown to be involved in transcriptional activation by methylating histone H3, the histone acetyltransferases CBP/p300 and possibly other proteins in the transcription initiation complex (reviewed in Lee *et al.*, 2005). Additionally, arginine methylation of DNA

polymerase β was reported to stimulate polymerase activity by enhancing DNA binding and processivity (El-Andaloussi *et al.*, 2006). A specific involvement in JAK/STAT signaling, in particular of STAT92E methylation, is therefore difficult to determine by the approaches described above.

The protein arginine methyltransferase CG9882/Dart7 was not expressed during embryonic and larval development at a level detectable experimentally (see 3.3.2; Boulanger *et al.*, 2004). Therefore, an involvement in JAK/STAT signaling at this time point seemed unlikely. However, it is possible that low expression levels, not detectable by *in situ* hybridization, are sufficient for functional activity of CG9882/Dart7. Low levels of the transcript were detected in the ovaries by Northern Blotting (Boulanger *et al.*, 2004). Additionally, *CG9927/Dart6* and *CG16840/Dart8* mRNA is predominantly found in ovaries (Boulanger *et al.*, 2004). However, an interaction of these Darts, in particular of CG9882/Dart7, with JAK/STAT signaling during oogenesis remains to be determined.

4.3.2 STAT92E^{R30mut}-GFP mutants

A function for arginine methylation specifically in JAK/STAT signaling or STAT92E arginine methylation itself could not be shown with the assays used (see Fig. 3.19, Fig. 3.21, Fig. 3.22 & Fig. 3.24). To differentiate between potential broad effects caused by inhibition of Darts and a specific involvement of arginine methylation in JAK/STAT signaling, the putative target residue for methylation of STAT92E was identified. STAT1 methylation at a highly conserved arginine residue within the N-terminal domain was reported recently (Mowen *et al.*, 2001). Alignments of several STAT proteins identified the corresponding arginine residue of the *Drosophila melanogaster* STAT92E protein at position 30 (see Fig. 3.23). Mutations of this residue did not alter the sub-cellular localization or the DNA binding ability of *Drosophila* STAT92E (see Fig. 3.25 & Fig. 3.26). However, expression levels of STAT92E^{R30mut}-GFP variants compared to STAT92E-GFP seemed to be reduced as judged by GFP fluorescence detected in transfected cultured cells. This might indicate that STAT92E^{R30mut}-GFP variants are less stable than STAT92E-GFP, a conclusion also made by others for STAT1 and STAT3 mutants (Meissner *et al.*, 2004; Komyod *et al.*, 2005). Consistently, STAT92E^{R30mut}-GFP mutants were not able to activate reporter gene expression to an extent similar to STAT92E-GFP (see Fig. 3.27). However, *in vivo* mis-expression of these mutant proteins resulted in dominant-negative phenotypes (see Fig. 3.28 & Fig. 3.29). It

is possible that these effects caused by STAT92E^{R30mut}-GFP variants was based on the binding at enhancer sites and the inability of subsequent activation of target gene expression. These mutant proteins might have lost the ability to interact with a subset of proteins resulting in the dominant-negative phenotype. Arginine methylation, for example, is often involved in protein-protein interactions. Therefore, disruption of the potential methylation target abolishing its modification might impede important interactions of STAT92E with the transcriptional machinery. As a result, target gene expression could not be activated to its full extent and DNA-bound STAT92E^{R30mut}-GFP dimers blocked the binding of endogenous wild type STAT92E. An alternative model suggests that arginine methylation of STAT1 prevents the binding of the negative regulator PIAS1 (Mowen *et al.*, 2001). Inhibition of arginine methylation led to an increased interaction of STAT1 with PIAS1 and resulted in reduced transcriptional response upon pathway stimulation (Mowen *et al.*, 2001). PIAS proteins can negatively regulate STAT activity at different levels in vertebrates. For example, PIAS1 specifically inhibits DNA binding of activated STAT1 (Liu *et al.*, 1998). However, *Drosophila* STAT92E^{R30mut}-GFP proteins were still able to bind to DNA (see Fig. 3.26). Another PIAS protein, PIASy, does not affect the DNA binding activity of STAT1. It rather acts as a transcriptional co-repressor of STAT1 (Liu *et al.*, 2001). In principal, a model, in which mutant STAT92E-GFP dimers are DNA-bound and negatively regulated by associated *Drosophila* PIAS, is therefore possible.

Beside reduced stability, structural changes in the STAT92E^{R30mut}-GFP mutant proteins independent of arginine methylation might have impaired interactions with co-factors. This could also have contributed to the inability of these mutant proteins to act as transcriptional activators. STAT92E-GFP variants were still able to translocate to the nucleus and bind to DNA following stimulation (see Fig. 3.25 & Fig. 3.26) regardless of these potential structural changes. Therefore, it seems possible that interactions with the transcriptional machinery are disturbed in these mutants, adversely affecting transcriptional initiation. It was shown, for example, that interaction of STAT2 or STAT1 with the histone acetyltransferases CBP/p300 (Bhattacharya *et al.*, 1996; Zhang *et al.*, 1996) is required for JAK/STAT-dependent transcriptional activation.

Structural changes in the N-terminal domain of STAT92E^{R30mut}-GFP variants could also affect important interaction between STAT dimer partners. It has been proposed that a conformational change of activated STAT dimers facilitate their dephosphorylation by presenting the phosphorylated tyrosine residue to phosphatases (Fig. 4.2; Zhong *et al.*, 2005). In this model, phosphorylated STAT dimers exist in a parallel structure. The conformational

rearrangement to an antiparallel dimeric structure after translocation from the DNA results in the exposure of the phosphorylated tyrosine to nuclear phosphatases (Zhong *et al.*, 2005). Mutation of the conserved R30 of *Drosophila* STAT92E may alter the structure of the N-terminal domain of this mutant protein. This, in turn, could affect the ability of STAT92E^{R30mut}-GFP proteins to form the antiparallel dimeric structure, which would interfere with dephosporylation. Phosphorylated STAT92E^{R30mut}-GFP dimers would remain in the nucleus and be again able to bind to DNA. Indeed, it was reported that the conserved arginine residue is located within an evolutionary conserved salt bridge network in the N-terminal domain (Vinkemeier *et al.*, 1998; Meissner *et al.*, 2004), which indicated an important structural role. The amino acids contributing to this network are conserved throughout most STAT proteins including *Drosophila* STAT92E. Therefore, it is very likely that R30 of STAT92E occupies a similar structural role.

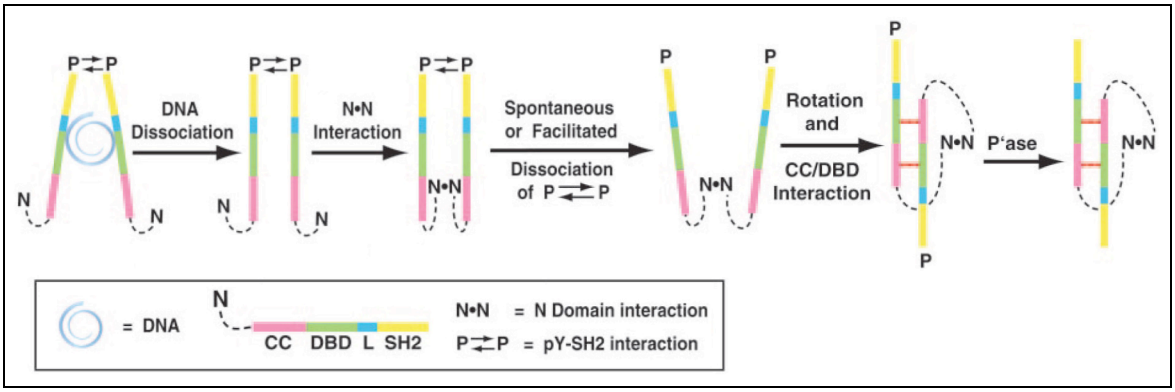

Fig. 4.2: Possible scenario for STAT inactivation (modified after Zhong *et al.*, 2005). Inactivation of STAT dimers requires translocation from the DNA. Interaction between the N-terminal domains (N) then disrupts the interaction between SH2 domains (SH2) and the phosphorylated tyrosine (P or pY) of the dimer partner. This structural rearrangement results in an anti-parallel structure of the dimer presenting the phosphorylated tyrosine residues to phosphatases (P´ase). Interactions between the coiled-coil (CC) and the DNA binding (DBD) domain are also required for this structural rearrangement. L: Linker domain.

A previous report suggested that R31A and R31E mutations of STAT1 resulted in increased JAK/STAT activity (Mowen *et al.*, 2001). However, STAT92E-GFP mutants carrying the mutations R30A, R30E or R30K did not have a positive regulatory effect on JAK/STAT signaling. R30 mutations of *Drosophila* STAT92E-GFP reduced the induction of reporter gene expression (see Fig. 3.27) and resulted in molecules that exhibited dominant-negative phenotypes *in vivo* (see Fig. 3.28 & Fig. 3.29). Although arginine mutations of STAT1 and STAT6 have been reported not to result in gain-of-function proteins (Chen *et al.*, 2004;

Meissner *et al.*, 2004; Komyod *et al.*, 2005) contrary to a previous report (Mowen *et al.*, 2001), these vertebrate STAT mutant proteins also behave differently from *Drosophila* STAT92E variants. Substitution of the conserved arginine residue resulted in vertebrate STAT proteins that were not able to accumulate in the nucleus upon pathway stimulation. In addition, no DNA binding of these mutants was detectable (Chen *et al.*, 2004; Meissner *et al.*, 2004; Komyod *et al.*, 2005). *Drosophila* STAT92E^{R30mut}-GFP variants showed a different functional behavior. These mutant proteins were able to translocate to the nucleus and bind to DNA under stimulated conditions (see Fig. 3.25 & Fig. 3.26). Sequence variations in the N-terminal domain between *Drosophila* STAT92E and vertebrate STATs could potentially reflect different demands on these proteins contributing to the observed functional deviation. In addition, this could also be an explanation for the dominant-negative phenotype of *Drosophila* STAT92E^{R30mut}-GFP compared to vertebrate STAT arginine mutants. *Drosophila* STAT92E^{R30mut}-GFP variants have the potential to directly interfere with transcriptional initiation, since they are still able to accumulate in the nucleus and to bind DNA, in contrast to vertebrate STAT mutants, which remain in the cytoplasm.

The results obtained in the present study suggest a general role for arginine methylation in transcriptional or translational regulation rather than a specific involvement in JAK/STAT signaling. Consistently, arginine methylation of STAT92E could not be detected by immunoprecipitation and Western blotting experiments (see Fig. 3.24). Recently, several reports investigated STAT protein methylation of the N-terminal conserved arginine residue (Meissner *et al.*, 2004; Komyod *et al.*, 2005). The utilized assays, including Mass spectroscopy, could not reveal a methylated arginine at the proposed position (Meissner *et al.*, 2004). Furthermore, 3D structural analysis is arguing against a post-translational modification of this arginine residue (Komyod *et al.*, 2005), because it is part of a salt bridge network that is conserved between distinct members of the STAT family (see above; Vinkemeier *et al.*, 1998).

Taken together, these results imply an important role of R30 within *Drosophila* STAT92E. However, it seems that methylation of this residue does not occur and thus does not contribute to full activation of STAT92E. Therefore, missing of R30 methylation cannot be the reason for STAT92E^{M647H}-GFP acting as a dominant-negative allele. This mutant protein has lost its ability to activate transcription due to a single amino acid substitution in its SH2 domain. Structural effects on the transactivation domain or interaction surfaces of STAT92E caused by this substitution might have contributed to its function as dominant-negative allele of

STAT92E. Recently, other modifications of STAT proteins have been described. Glycosylation of a N-terminal located threonine residue of STAT5 was shown to to be required for binding to the transcriptional co-activator CBP (Gewinner *et al.*, 2004). Additionally, this co-activator CBP was reported to acetylate a lysine residue in the transactivation domain of STAT3 (Wang *et al.*, 2005) stimulating sequence-specific DNA binding and transactivation activity of STAT3. Thus, STAT activation is not only controlled by its state of phosphorylation, but cooperation of different modifications of STAT proteins is necessary for complete activation and recruitment of co-factors. Although it is not known if *Drosophila* STAT92E is modified in a similar way, it is possible that both R30 and M647 substitutions interfere with potential modifications and thus affect the recruitment and interaction with positively or negatively regulating co-factors. This, in turn, could have an impact on transcriptional activation mediated by *Drosophila* STAT92E resulting in dominant-negative over-expression phenotypes *in vivo*.

4.4 Outlook

The regulation of JAK/STAT signal transduction is an important step in the development of an organism. Additionally, mis-regulation has been shown to be involved in multiple diseases. Analysis of post-translational modifications of STAT proteins, in addition to tyrosine phosphorylation, in the context of the regulation of JAK/STAT activity has not been a major focus up to now. However, several modifications of STAT, including acetylation (Wang *et al.*, 2005) and glycosylation (Gewinner *et al.*, 2004), have recently been described to affect JAK/STAT pathway activity. The present as well as further studies examining the role of STAT modifications in signaling will help to elucidate their respective effects on signal transduction by JAK/STAT. Additionally, generation and analysis of other mutant STAT proteins, in particular of constitutively active alleles, will add to the comprehension of STAT function. Furthermore, such mutants would also be a potentially valuable tool for *in vivo* epistatic analysis of JAK/STAT signaling regulators and inter-pathway crosstalk. Ultimately, these findings will also contribute to the understanding of diseases caused by JAK/STAT mis-regulation and could lead to novel approaches for the treatment of those diseases.

5 Summary

The highly conserved JAK/STAT signal transduction cascade is required for multiple developmental and cellular processes. In mammals pathway activation by binding of a multitude of ligands to their respective receptors results in phosphorylation of STAT transcription factors at a highly conserved tyrosine (Y) by JAKs. This modification is essential and was thought to be sufficient for STAT activation, since Y mutation completely abolishes STAT function.

In *Drosophila* the JAK/STAT pathway has a greatly reduced complexity and redundancy simplifying the functional analysis of STAT activation. Therefore, *Drosophila* STAT92E function in cell culture and *in vivo* assays was investigated by introducing mutations into STAT92E based on previously reported vertebrate alleles. Constitutive Y phosphorylation of the STAT92E^{M647H}-GFP mutant is sufficient for nuclear accumulation and DNA binding even under non-stimulated conditions. However, no transcriptional activation could be detected. Additionally, STAT92E^{M647H}-GFP causes dominant-negative phenotypes when over-expressed *in vivo*. This data demonstrated that Y phosphorylation alone is not sufficient for complete STAT92E activation suggesting the presence of another regulatory step. A potential mechanism is the reported methylation of a conserved arginine (R) of murine STAT1. This is further supported by detected interactions of the annotated *Drosophila* Protein R Methyltransferase (Dart) 7 with JAK/STAT pathway components in yeast two-hybrid screens. To assess a regulatory role of methylation on STAT activity, putative *Darts* were targeted in RNAi knockdown experiments. Additionally, mutant STAT92E^{R30mut}-GFP variants were generated, which caused dominant-negative over-expression phenotypes *in vivo*. However, since STAT92E R methylation could not be detected, it seems more likely that mutation of the conserved R affects interaction surfaces disrupting protein-protein interactions required for transcriptional initiation. Furthermore, the results of the RNAi experiments and cell culture assays and results of others suggest that protein methylation affects general transcriptional control, rather than that it has a specific effect on JAK/STAT components.

The results of this study demonstrate the evolutionary conserved crucial role of Y phosphorylation for STAT activity. Nevertheless, this modification is not sufficient for activation suggesting additional regulatory mechanisms. Further analysis of STAT variants carrying mutations in potential modification sites will help to elucidate STAT functionality and thus provide useful information for the treatment of diseases caused by JAK/STAT pathway mis-regulation.

6 References

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